Splenic Antigen Presenting Cells and Their Role in Shaping The Immune Response

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CHAPTER I

INTRODUCTION

THE IMMUNE SYSTEM

The immune represents a highly versatile defense system that evolved to protect vertebrates from invading pathogens and cancer. Cells and molecules of the immune system are able to specifically recognize and eliminate an apparently unlimited variety of foreign invaders. They act together in a dynamic network and its dysregulation may give rise to a series of diseases (1).

In mammals, the immune system includes innate and adaptive components. The innate immune system consists of soluble effector molecules and cellular components like myeloid cells, while the adaptive immune system is constituted by antibody producing B cells as well as $CD4^+$ helper and $CD8^+$ killer T cells. Both systems act in close cooperation to protect the host against pathogens (1, 2).

Generally, the innate immune system is considered to act nonspecifically. It provides the first line of defense against infection. Elements of the innate immune system are present before the onset of infections and they are not specific to particular pathogen but rather recognize classes of molecules peculiar to frequently encountered pathogens (1-3). In contrast, the adaptive immune system is thought of as being very specific. This system is capable of recognizing and selectively eliminating specific foreign microorganisms and molecules. Moreover the adaptive immune system reacts faster and stronger upon re-encounter of the same pathogen i.e. it exhibits immunological memory (1-3).

The adaptive immune system is coming into play when the innate immune system is overwhelmed by an infection, although many infections are solely cleared by innate immune system. A few of the pathogens can overcome innate immune mechanisms and adaptive immune system is essential for defense against such invaders. Therefore, both innate and adaptive components have to be fully synchronized. The adaptive immune system requires instruction from the innate immune system indicating the origin and nature of the antigen they recognize. For instance, T cells are unable to recognize antigen directly. They require antigen to be processed and presented by so-called antigen presenting cells. This is so-called innate control of adaptive immune responses and will be described extensively below (1-3).

1. INNATE IMMUNE SYSTEM

The innate immune system consists of soluble molecules as well as of cellular components. Soluble factors can be constitutively present in circulation; some of them can also be induced upon infection. Cells of the innate immune system are mainly phagocytic, such as macrophages and neutrophils. Very important compounds of the innate immune system are also anatomical barriers such as skin (1-6).

1.1 SOLUBLE MOLECULES

A few soluble components will be described here that are essential for the innate and activation of the adaptive immune response. They display local as well as long range activities.

1.1.1 The Complement System

The complement system consists of more than 35 proteins. It is found constitutively in circulation. The main biological functions of complement are defense against infections and clearance of debris such as apoptotic and necrotic cells (1, 7, 8). Complement is activated by three different pathways: classical, lectin and alternative (Fig. 1.1). They share the common step of activating the central component C3, but they differ according to the nature of activation. The classical pathway is activated after formation of antibody/antigen complexes, the lectin pathway is activated after the recognition and binding of pathogen-associated molecular patterns (PAMPs) by lectin proteins, the alternative pathway is continuously „on" due to spontaneous activation of C3 (1, 7, 8). Activation of complement results in a cascade of enzymatic reactions that finally leads to a so-called membrane attack complex, which than forms pores in the membrane of the susceptible target (1, 7, 8). Many of the complement components are activated by proteolytic cleavage. The resulting peptides especially from C3 and C5 act as an important chemoattractants called anaphylatoxins that attract cells of the innate immune system. The complement system is an important part of innate immunity but also participates in antibody response of the adaptive immune system (8, 9).

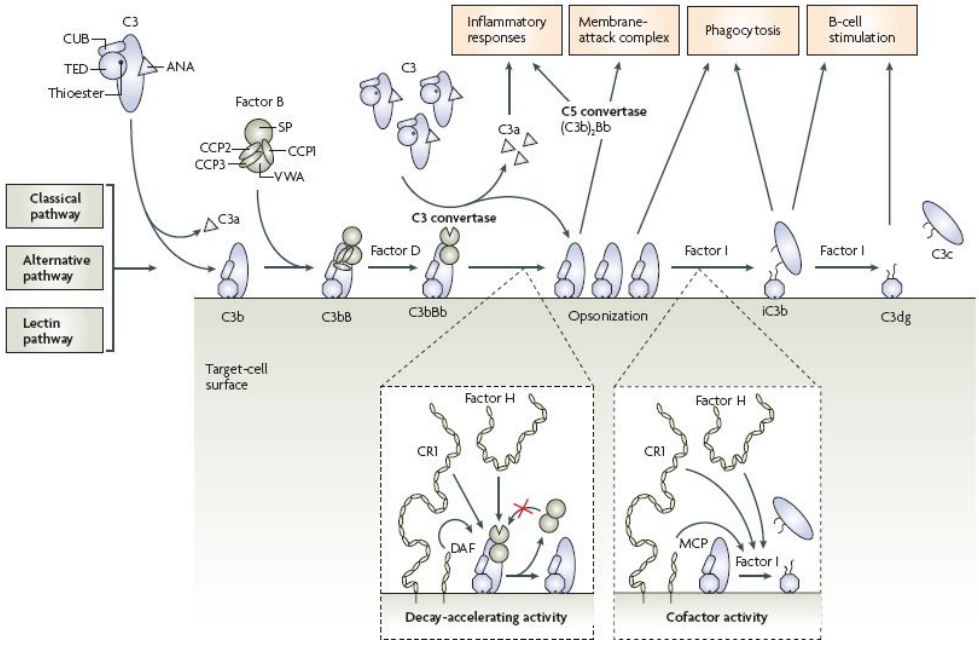


Figure 1.1 Three pathways of the complement system (10)

1.1.2 Type I Interferons

Type I Interferons (IFNs) were discovered in 1957 by Isaacs and Lindenmann (11) and constitute one of the most important classes of cytokines. They are amongst the first to be induced during infection. Type I IFNs belong to the class II family of α-helical cytokines and constitute a multi-member cytokine family (13 subtypes of IFN-α, one IFN-β, -ε, -κ, and several -ω, -σ and –τ). IFN-σ and IFN-τ are absent in humans, IFN-τ was found only in ruminants. IFN-ω is closely related to IFN-α. IFN-ε is expressed in the placenta, therefore may play a role in reproduction, IFN-κ is expressed by keranocites.

While for many of the isotypes of IFNs the function is not yet clear, for immunologists IFNs-α and IFN-β are the main isotypes of interest and will be taken from now on synonymously for type I IFNs (1, 12-15). The grouping of IFN-α and IFN-β together as a type I IFNs is due to the fact that they display similar amino acid sequences and structures and namely due to that they share the same receptor – type I IFN receptor (IFNAR). IFNAR is a ubiquitously expressed heterodimeric receptor composed of IFNAR1 and IFNAR2 chains. IFNAR2 serves as the ligand binding chain, yet both chains are required for signal transduction (1, 12-15).

IFN-β is a master type I IFNs. For instance, it is induced as first IFN upon stimulation of toll-like receptor 3 (TLR3) or toll-like receptor (TLR4), and acts in an autocrine and paracrine fashion to induce other type I IFNs isotypes (12, 15, 16). In details, binding to the IFNAR receptor results in cross-activation of two receptor associated tyrosine kinases – Jak1 and Tyk2, followed by activation of two members of the family of signal transducers and activators of transcription (STATs) – STAT1 and STAT2. Further heterodimers of STAT1/STAT2 associate with the IFN regulatory factor 9 (IRF-9) to form the complex IFN-stimulated gene factor 3 (ISGF3). The ISGF3 can initiate transcription by binding to the upstream regulatory consensus sequences of IFN-α/β inducible genes (IFN-stimulated response elements, ISRE) (10, 15). Other IRF family members, IRF3 and IRF7 are required to initiate and amplify the expression of type I IFNs (16).

Beyond the well established role of type I IFNs in protection against viral and bacterial infections, they are also spontaneously produced under steady state conditions (16, 17). Low, constitutive levels of type I IFNs are believed to prime the immune system and thus are prerequisite for enhancement of IFN-α/β production subsequent to infections. They serve as a so-called „weak signal for strong responses" (16-18). The mechanism of spontaneous production remains still unknown. Possibly, IRF-3 which is constitutively expressed induces very low levels of IFN-α/β, resulting in a weak signal through IFNAR receptor that would lead to ISGF3 dependent IRF-7 expression. Due to this fact upon viral infection both IRF-3 and IRF-7 are already present and ready to rapidly amplify the production of type I IFNs (Fig. 1.2) (16).

Type I IFNs are highly pleiotropic cytokines. They engage a well established position as part of the innate immune system. In addition they have been shown as potent regulators of adaptive immunity more recently. They exert broad regulatory effects and various cell types are strongly affected by these cytokines (13, 19-21). For instance type I IFNs produced primarily by plasmacytoid dendritic cells (pDCs) during viral infection (22) stimulate maturation and activation of conventional dendritic cells (cDCs) and enhance antigen presentation (23-25). They exert both direct and indirect effects on T cells and play a key role in the fine-tuning of their activity by delivering both stimulatory and inhibitory signals (26). Moreover, type I IFNs are powerful polyclonal B-cell activators that induce a strong primary humoral immune response (14).

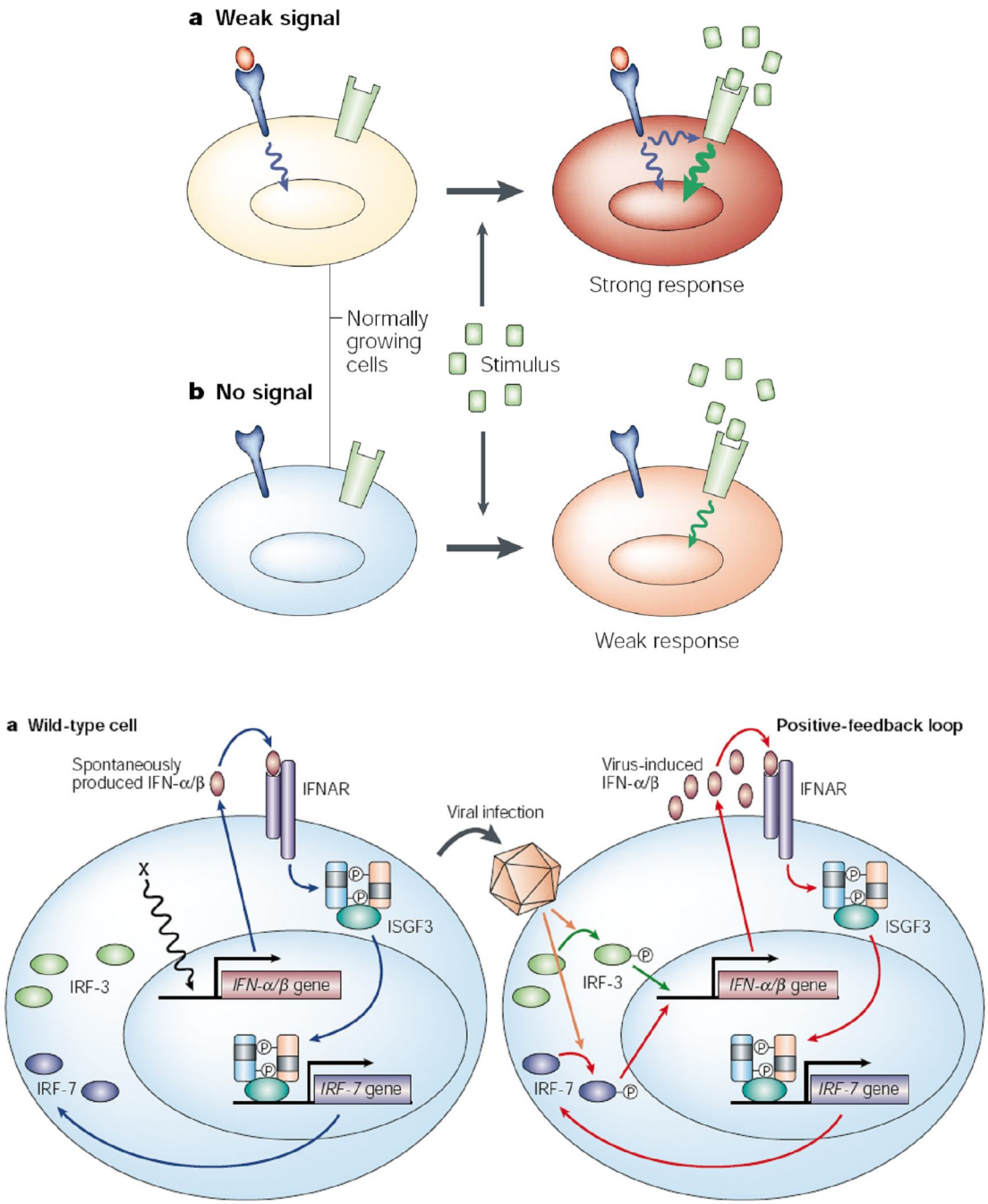


Figure 1.2 Week IFN-α/β signal is necessary for its amplification during antiviral responses (16)

1.2 CELLULAR COMPONENTS OF THE INNATE IMMUNE SYSTEM

Almost every cell in the body is able to exert a defense reaction against pathogens and could be considered as a part of the immune system. Especially epithelial tissues like the epidermis of the skin and the mucosal tissues serve as a barrier between the body and environment. They should be considered as a first line of defense, protecting the internal tissues from environmental stresses, chemical damage and bacterial infection. Epithelia of the skin and oral mucosa are examples of the toughest and most protective epithelia (1, 27). Although, mucosal epithelia represent the major site of entry of pathogens, they are also responsible for a symbiotic relationship with bacterial microflora in the gut. Beside such cells the innate immune system includes cells that are specialized for certain functions in the immune reactions.

1.2.1 Cells of the innate immune system

Once pathogens overcome the epithelial barrier and replicate in the tissues of the host, they are usually rapidly recognized by migratory phagocytic and non-phagocytic cells of the innate immune system. To this group of cells belong blood monocytes, macrophages, dendritic cells, neutrophilic, eosinophilic and basophilic granulocytes as well as mast cells. Natural killer (NK) cells are also considered as a part of the innate immune system. They do not exhibit pahgocytic properties.
Phagocytic cells of the innate immune system recognize pathogens by means of cell surface receptors and engulf them by a process called phagocytosis. In most cases this is followed by death of the pathogen. Since a major part of this thesis is concerned with dendritic cells they will be described more extensively below.

1.2.2 Dendritic cells

Dendritic cells (DCs) were identified in 1973 by Ralph M. Steinman (28). They were described as a family of cells that regulate the immune response. Dendritic cells acquired their name because they possess long membrane extensions that resemble the dendrites of nerve cells. Together with B cells and macrophages they are considered as professional antigen presenting cells (APCs) for T cells, although DCs are the most

efficient APCs. At the moment they appear to be an extremely heterogenous family of cells, yet all of them constitutively express MHC class II molecules (MHC II, see description below) (1, 29). At steady state DCs are phenotypically and functionally immature. After exposure to microbial products or inflammatory stimuli, DCs undergo an activation program, generally referred to as "maturation". This process induces major phenotypic and functional modifications, affecting especially antigen capture, processing and intracellular MHC II trafficking. Mature DCs down-regulate their ability to take up newly encountered antigens. They also down-regulate their intracellular levels of MHC II while surface levels of MHC II and co-stimulatory molecules are dramatically up-regulated (Fig. 1.3) (29-31).

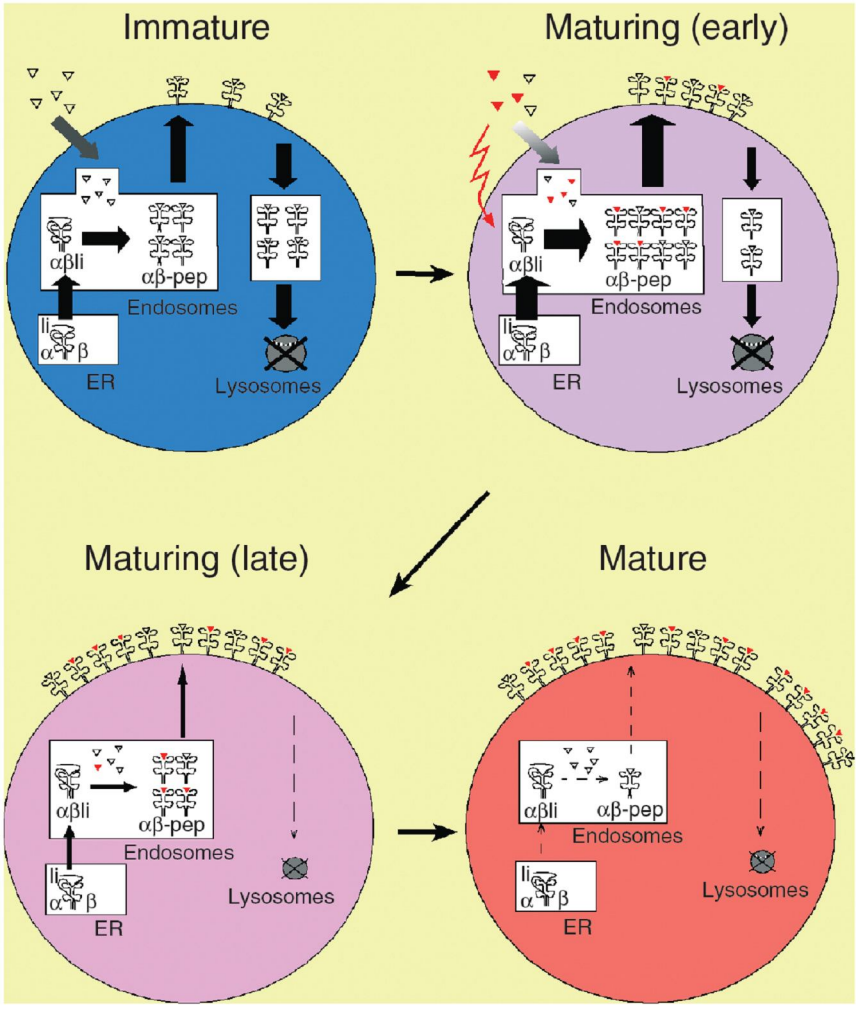


Figure 1.3 Maturation and control of MHC II antigen presentation in dendritic cells (32)

1.2.2.1 Diversity of DC subsets

Generally the dendritic cell family consists of conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs). Further cDCs can be subdivided into non-lymphoid tissue migratory and lymphoid tissue resident DCs. The major function of conventional DCs is maintenance of self-tolerance in steady state and induction of specific immune responses against invading pathogens. In contrast plasmacytoid DCs are also called "interferon-producing cells" and their main function is to secrete high amounts of type I IFNs in response to viral infections (1, 29).

DCs in non-lymphoid tissues. Here, one has to mention DCs that are present in sterile tissues like pancreas and heart, DCs present in filtering sites such as liver and kidney, and finally DCs present at environmental interfaces like lungs, gut and skin. Among all these diverse DCs epidermal DCs also called Langerhans Cells (LCs) are the most studied and the best characterized population.

Langerhans Cells were first detected in the skin by Paul Langerhans already in 1868 (33). At that time their role was not appreciated and they were rather considered to belong to the nervous system due to their morphology. LCs constitutively express high levels of MHC II molecules and also the lectin langerin (CD207), forming the intracytoplasmic Birbeck granules. However, recently it was shown that expression of langerin is not only specific for LCs (34, 35). In mice, it is also expressed at low levels on some migratory DCs in lymphoid organs and on some DCs populations present in lungs and dermis.

Tissue DCs sample antigens from the environment and migrate constantly through afferent lymphatics to the T cell areas of draining lymph nodes (LN). Here they present acquired antigen to T and B cells (1, 29, 30, 36).

DCs in lymphoid tissues. Five major DC subsets have been identified in lymphoid tissues of uninfected laboratory mice. Generally they all express high levels of CD11c molecule (the α chain of integrin CR4) and MHC II molecules. Four other markers can be used additionally to further define DCs subpopulations: CD4 (a member of the immunoglobulin superfamily and co-receptor for MHC II mediated activation of T cells), CD8α (a membrane bound dimer of two α chains, also a member of the

immunoglobulin superfamily and co-receptor for MHC I mediated activation of T cells), CD11b (the α chain of MAC-1 integrin) and CD205 (DEC-205, a C-type lectin).

In spleen all DCs express CD11c and MHC II. They can be further divided on 2 major subpopulations: $CD4^+CD8\alpha^-CD11b^+$ (shortly called also $CD8\alpha^-$ DCs). Recently it was shown that this population also expresses the antigen recognized by the 33D1 monoclonal antibody – $CD8\alpha^-33D1^+$ (37). The second population is $CD4^-$ $CD8\alpha^+CD11b^-$ (shortly called also $CD8\alpha^+$ DCs). This population additionally expresses CD205 molecule. $CD4^-CD8\alpha^-CD11b^+$ DCs have been also described and called shortly double negative DCs. The particular subpopulations also differ by their localization in the spleen. The majority of $CD8\alpha^-$ DCs are found in the marginal zone, whereas $CD8\alpha^+$ DCs are present in T cell areas (1, 29, 30, 36).

DCs found in the LNs are more heterogenous as they include additional migratory subpopulation. Therefore, in the LN are present: $CD8\alpha^+$ DCs, $CD8\alpha^-$ DCs, double negative DCs and migratory DCs, which enter *via* afferent lymphatics. About one third of DCs from LN are migratory DCs. Generally, they were shown to exhibit following phenotype: $CD4^-CD8\alpha^{low(lo)}CD11b^+$ and $CD205^{high(hi)}$. Such migratory DCs may also express additional markers, which vary according to the site drained by the LN. For example migratory dermal DCs will express langerin (CD207) and they can be found in subcutaneous LN (1, 29, 30, 36).

DCs present in mucosa-associated lymphoid tissues like Peyer patches and isolated lymphoid follicles in the small intestine or in isolated follicles and appendix in the large intestine phenotypically resemble splenic DCs.

Thymic DCs localize in the medulla and the majority of them express CD8α. A minor subpopulation is $CD8\alpha^-$ and displays the signal regulatory protein-α (sirp-α). Thymic DCs play a major role in negative selection of T cells, i.e. in the induction of central tolerance (29, 30).

Plasmacytoid DCs (pDCs). Mouse pDCs has been indentified recently (38-41). These cells have a unique surface phenotype: $CD45RA^{hi}B220^+CD11c^{lo}CD11b^-MHC\ II^{lo}$. Additionally, they express the PDCA1 and siglec-H molecules, recently identified as a specific surface markers for mouse pDCs (42). Mouse pDCs are known to produce large amounts of type I IFNs in response to viral stimulation. Therefore they are also called “interferon producing cells” (IPCs) (29).

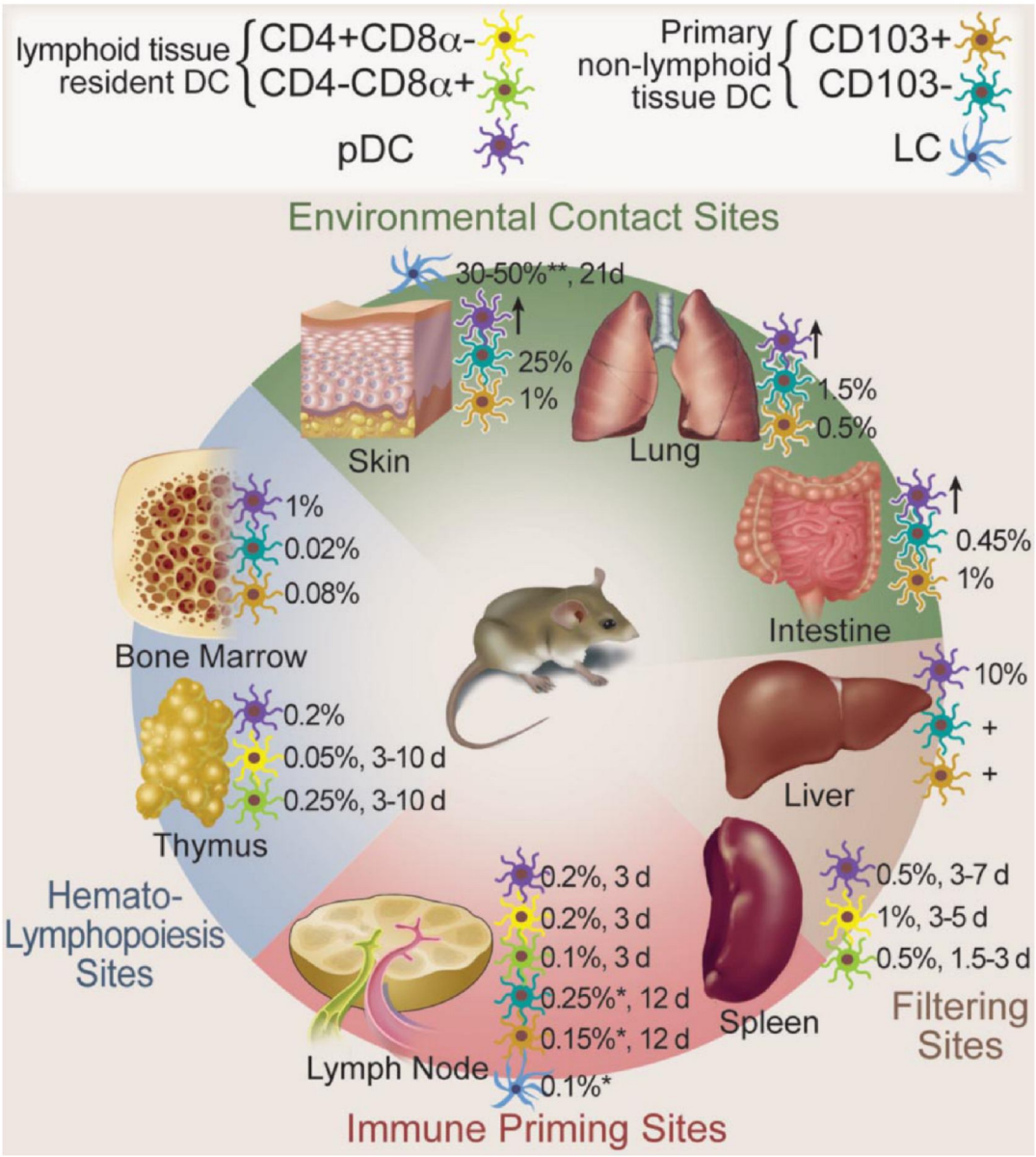


Figure 1.4 Mouse DC populations (29)

1.2.2.2 Origin of DCs

DCs originate from hematopoietic stem cells (HSCs) present in the bone marrow. Here, the self-renewing HSCs give rise to two major developmental pathways of DCs: lymphoid and myeloid. Initially it has been suggested, that DCs are exclusively of myeloid origin, since they share many features with monocytes and macrophages (43). Now it is known that both the common myeloid progenitor (CMP) as well as the common lymphoid progenitor (CLP) from bone marrow can give a rise to DCs. (44-46). Moreover, by several experimental approaches it has been shown that both CMP

and CLP can differentiate into all types of DCs indicating the plasticity of the hematopoetic development (43, 47). Almost all early DCs precursors have been found to express FMS-related tyrosine kinase 3 (Flt-3) (29). An additional intermediate precursor was described that lacks lineage specific markers- lin^-, and expresses the markers CX_3CR1 and CD117. This precursor is common to both macrophages and DCs, it was shown to be able to differentiate into cDCs but not into pDCs, which indicates the branch for pDCs development (43, 48, 49). Finally the late DCs precursors express already CD11c, the marker that is common to all DCs. They still lack surface MHC II expression. In the bone marrow very few developed cDC are found since the final differentiation normally takes place in peripheral organs (43, 49).

1.2.2.3 Differential antigen presentation capacity of DC subsets

Several studies have revealed that particular subpopulations of DCs display specialized antigen presenting abilities (53, 54). Especially only $CD8\alpha^+$ DCs possess the machinery to efficiently cross-present antigens, i.e. to present exogenous antigens *via* MHC I) *in vivo* (37, 50). Similarly, *ex vivo* isolated, tissue resident $CD8\alpha^+$ DCs were shown to be the most efficient population in cross-presentation experiments. Nevertheless, $CD8\alpha^+$ DCs can also efficiently present antigen *via* MHC II molecule and activate $CD4^+$ T cells. The role of $CD8\alpha^-$ DCs is less well understood. Tissue resident $CD8\alpha^-$ DCs can generally cross-present antigens, but are much less efficient in comparison to $CD8\alpha^+$ DCs. Their role in antigen presentation to $CD4^+$ T cells *via* MHC II molecules is much more prominent (31, 51). Double negative DCs were shown to be functionally very similar to $CD8\alpha^-$ DCs (50). Non-lymphoid tissue migratory DCs were found to play a crucial role in the presentation of antigens present in the peripheral tissues (31, 51). And finally, pDCs are generally considered as poor presenters of exogenous antigens but they can efficiently present endogenous antigens for instance during viral infections (31, 52, 53).

1.3 RECOGNITION OF NON-SELF MOLECULES BY THE INNATE IMMUNE SYSTEM

Innate immune antigen recognition (also called pattern recognition) relies on a limited number of germline-encoded receptors (2, 5, 6). This recognition is based on the detection of molecular structures that are unique to microorganisms. Each host receptor, called also pathogen recognition receptor (PRR) can bind to a large number of molecules that have a common structural motif or pattern, so-called pathogen-associated molecular patterns (PAMPs). Bacterial PAMPs are often components of the cell wall, such as lipopolysaccharide, peptidoglycan, lipoteichioc acid and cell wall lipoproteins. Thus, the detection of these structures by the innate immune system signals the presence of microorganism. The recognition of viruses also follows this principle. Here, however targets for immune recognitions usually are viral nucleic acids or replication intermediates (2, 5, 6, 54).

The family of Toll-like receptors (TLRs) is the major and the most extensively studied class of PRRs. TLRs can be divided into subfamilies recognizing related PAMPs: TLR2 as heterodimer with TLR1 or TLR6, and TLR4 recognize glycolipids, TLR3, TLR7, TLR8 and TLR9 recognize nucleic acids. The most important cell types expressing TLRs are APCs including DCs, macrophages and B cells (1, 2, 5, 6, 54).

C-type lectin receptors (CLR) are another group of proteins involved in innate recognition. These receptors recognize carbohydrate structures on cell wall components of microorganisms. One of their major functions is to internalize antigens for lysosomal degradation. To this family of receptors belong molecules like: mannose receptor (MR, CD206), DEC-205 (CD205), dectin-1, langerin (CD207), DC-SIGN (CD209), SIGN-R1 (CD209b) (55, 56).

Another class of PRRs is found intracytosolic and includes nucleotide-binding oligomerization domain proteins (NOD) and RIG-I-like receptors (RLR). Such receptors are responsible for the recognition of viral infections but are also activated by certain bacteria or even small molecules (1, 2, 5, 6, 54).

Triggering of such receptors usually results in activation of signaling pathways that include NFκB and MAP kinases. This results in the induction of defense mechanisms like induction of type I IFNs or proinflammatory cytokines as well as a general activation of the cells of the immune system (57).

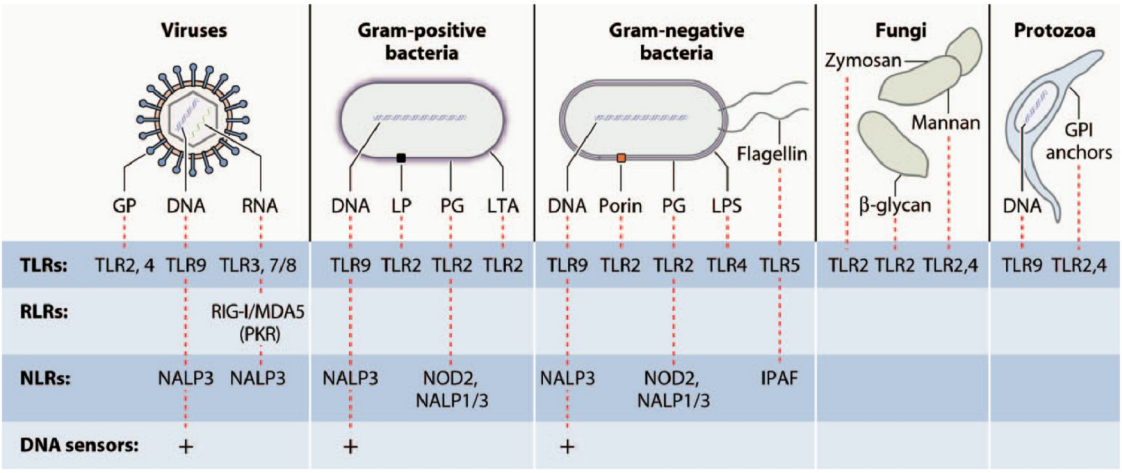


Figure 1.5 Innate recognition receptors – overview (6)

2. ADAPTIVE IMMUNE SYSTEM

In most cases the innate immune reaction is sufficient to control and clear the pathogen. Yet, some of the invaders developed sophisticated evasion mechanisms, such that a more specific response is required to defeat them. This is achieved by effector mechanisms of the adaptive immune system. The adaptive immune system is capable of recognizing and selectively eliminating specific foreign antigens. It is not independent of the innate immune system. Phagocytic cells and soluble molecules belonging to the innate immune system are particularly involved in activating and directing the specific immune response. This highly regulated interplay of innate and adaptive immune systems ensures, the most efficient way of eliminating foreign invaders (1).

2.1 CELLS OF THE ADAPTIVE IMMUNE SYSTEM

Lymphocytes are the cells constituting the adaptive immune system. Two major types of lymphocytes exist – T lymphocytes (T cells) and B lymphocytes (B cells). Lymphocytes are one of the many types of white blood cells produced in the bone marrow in the process of hematopoesis (1). T cells received their name because they further develop in thymus from precursors generated in the bone marrow, whereas B

cells are named according to their organ of origin in birds, the Bursa of Fabricii. In mammals they develop in bone marrow.

2.1.1 B cells

The most important task of B cells is to produce antibodies, thus they are responsible for humoral immunity. Primarily they are produced in fetal liver before birth and in bone marrow afterwards. B cells express antibodies as antigen-binding receptor on their membrane, the so-called B cell receptor (BCR). The BCR consists of two identical heavy and two identical light chains. While the C-terminal region of both chains is constant, the N-terminal region displays a high variability of sequences. Such variable regions are encoded in germline by several gene segments (V, D, J for heavy chain and V, J for light chain). During B cell development, the variable region (V) of both chains is assembled by rearrangement of the particular gene segments at the DNA level thus providing the basis for the extensive diversity of the BCR (1).

Binding of antigen to BCR together with appropriate help of T cells, causes B cells to rapidly divide and differentiate into memory B cells or plasma cells. Plasma cells represent effector B cells, which can produce vast amounts of antibodies in a secreted form, while memory B cells are the basis for the long lasting immunity against pathogens that an individual had encountered already once before. Besides their task to produce antibodies, B cells also are able to present antigen to T cells and produce immunoregulatory cytokines (58, 59).

B cells can be subdivided into several subpopulations. B2 cells are the major population in the circulation and spleen. They are responsible for highly specific antibody responses and the formation of the memory. B1 cells dominate the body cavities and are responsible for the production of natural antibodies (1, 60, 61). In the spleen there is also special B cell population, so-called marginal zone (MZ) B cells, which is described in more details in the next section.

2.1.1.1 Marginal Zone B cells

MZ B cells are uniquely located within the MZ of the spleen (Fig. 1.6) where the splenic arterioles open into a sinus and allow contact with blood. Generally MZ B

cells are responsible for early antibody responses to blood-borne pathogens that have entered the spleen. This response is fast and efficient, because MZ B cells have a reduced threshold for activation, proliferation and differentiation into antibody-secreting cells compared to other B cells (60-63). Although MZ B cells are defined primarily on the basis of their special anatomical location, the expression of a number of surface markers can be used to characterize these cells in more details.

MZ B cells express high levels of IgM, very low levels of IgD and CD23 (low affinity receptor for IgE). They also express high levels of CD21 (complement receptor type II), CD1d (MHC class Ib protein responsible for presentation of lipid antigens), CD9 (a scavenger receptor family protein) and CD25 (the α chain of IL-2 receptor). In addition, MZ B cells express high levels of co-stimulatory molecules, therefore they are often described as having an "activated" phenotype (62, 63).

MZ B cells were shown to play an important role in activation of other immune cells and regulation of their functions. This is mainly due to their special anatomical location, which allows them to have close contact with T cells, dendritic cells and NKT cells (64).

Importantly, MZ B cells have been recently shown to be not strictly confined to the MZ as believed before (65). They continuously shuttle between MZ and follicles in the white pulp of the spleen. This feature of MZ B cells facilitates transport of the blood-borne antigens to the DCs present in the follicles. Subsequently such DCs assist in the maturation of B cells by presentation of an intact antigen. In addition continuous shuttling of MZ B cells between MZ and white pulp follicles ensures efficient transport of T cell independent, blood derived antigens to follicular DCs and B cells (66).

2.1.2 T cells

The second major lymphocyte population, the T cells are also derived from bone marrow precursors. They migrate to the thymus where they undergo final differentiation. During maturation T cells express a unique-antigen binding heterodimeric molecule, the so-called T cell receptor (TCR) on their membrane, consisting of chains of equal size. Similar to the BCR, the two chains consist of a variable and a constant region and the variable parts are formed during ontogeny in the thymus by DNA rearrangement of particular gene segments. Once matured, T cells

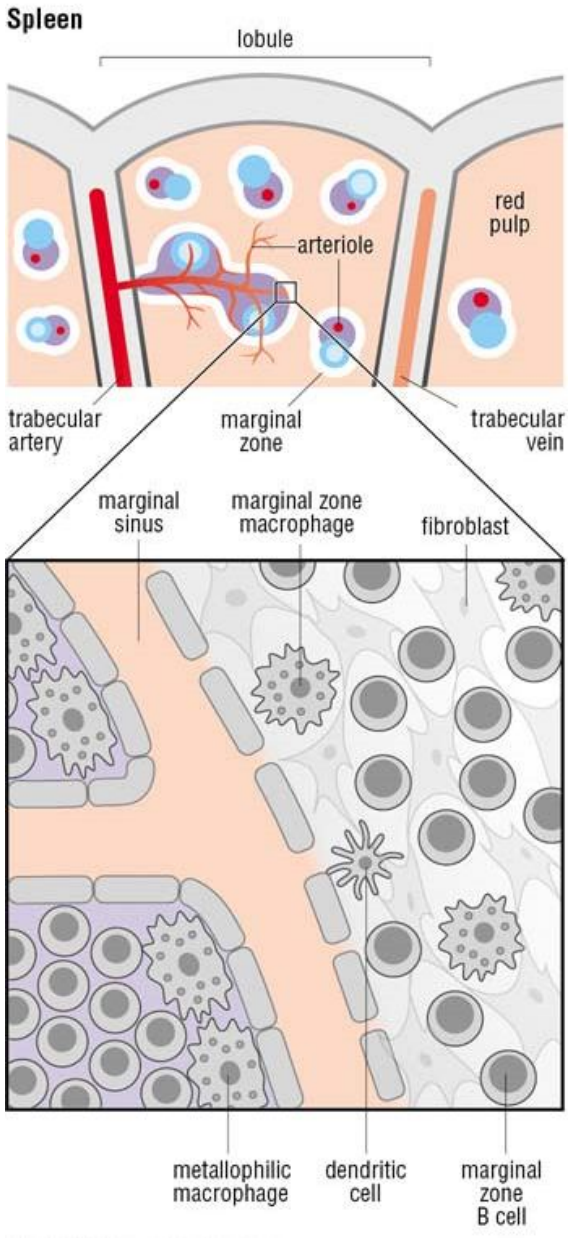


Figure 1.6 Particular anatomical location of the MZ B cells (172)

emigrate from the thymus and remain in circulation or migrate into the secondary lymphoid organs like spleen or LN.

T cells can be divided into two major subclasses, according to cell surface receptor expression. The majority of T cells express TCRs consisting of α and β chains, and the second group express TCRs made of γ and δ chains. The function of the latter T cell group is still enigmatic.

Among T cells which express α/β TCR there are two major subclasses, $CD4^+$ and $CD8^+$ T cells. They are different in the way they recognize antigen and by their effector functions (1, 67, 68). $CD4^+$ T cells recognize antigen presented *via* MHC II

and $CD8^+$ T cells recognize antigen presented *via* MHC I. $CD4^+$ T cells function as a so-called T helper cells (T_H), i.e. by cell cell contact or secretion of cytokines they aid other immune cells like macrophages, DCs or B cells to differentiate. As a consequence of priming T_H cells can develop into T_H1 cells (which mainly produce IFN-γ and lymphotoxin), T_H2 (which produce IL-4, IL-13, IL-5, IL-6 and IL-10), T_H17 (which produce IL-17) (1, 67-69). T_H1 cells are inducers of cellular immune responses, for example they enhance activity of macrophages. T_H2 cells help B cells to develop into antibody producing plasma cells and regulate the isotype switch that allows B cells to change the C-terminal constant region. T_H17 cells can efficiently recruit granulocytes into the sites of inflammation (69, 70).

Another recently discovered $CD4^+$ T cell population is so-called regulatory T cells (T_{reg}) population. They express the forkhead box P3 (Foxp3) transcription factor and CD25 (α chain of IL-2 receptor). T_{reg} play a crucial role in maintenance of tolerance and in regulation of immune responses in general (71).

$CD8^+$ T cells are also called cytotoxic or killer T cells. They are particularly involved in fighting against intracellular pathogens. After activation they are capable to efficiently lyse target cells. To eliminate infected cells they are using two principal mechanisms, one is perforin dependent, one Fas/FasL dependent. $CD8^+$ T cells can also interfere with infections by producing cytokines like IFN-γ or TNF-α (1, 68).

2.1.3 NKT cells

NKT cells were independently identified as a separate lineage of T cells. They are generally defined as T cells expressing NK lineage receptors, including NK1.1. At the same time they express a semi-invariant CD1d (see description below) restricted α/β TCR (72-75). The TCR of such NKT cells in most cases consist of Vα14-Jα18/Vβ8, Vβ7, Vβ2 chains in mice. Therefore, these cells are often called invariant Vα14 NKT cells. Ligands for activation of NKT cells *via* CD1d molecules include: α-galactosylceramide (αGalCer) from a marine sponge *Agelas mauritianus*, microbial ligands derived from Gram-negative, LPS negative members of the class of α-proteobacteria *Sphingomonas sp.*, and self ligands like isoglobotrihexosylceramide (iGb3).

Once NKT cells recognize lipid antigen presented by CD1d molecules they rapidly start within hour to massively produce cytokines like IL-4 or IFN-γ. This is quite in contrast to normal T_H cells, which are permanently driven into either IFN-γ producing T_H1 cells or IL-4 producing T_H2 cells. The contrary outcome of rapid cytokine production (T_H1 versus T_H2 response) is only partially understood.

Additional functions on NKT cells are numerous and they include roles in clearing infections, autoimmune disorders, allergic responses and tumor immunity (75). When clearing an infection NKT cells may act directly by lysing infected cells or through production of cytokines that enhance the innate response and indirectly drive adaptive immune responses. In tumor immunity NKT cells may kill CD1d-expressing tumor cells directly or indirectly through the activation of NK cells. Moreover NKT cells can provide direct help to B cells for antibody production. A cross-talk between DCs and NKT cells also takes place: NKT cells rapidly secrete T_H1 or T_H2 cytokines which might activate DCs to prime $CD4^+$ and $CD8^+$ T cells responses, driving them into particular direction depending on the cytokines produced (Fig. 1.7) (72).

NKT cells can be also activated indirectly *via* cytokines produced by APCs. For example, triggering of TLR signaling can lead to secretion of IL-12 by DCs, which in turn enhances the ability of NKT cells to present endogenous lipid ligands, like iGb3, independent of TCR triggering (72).

2.2 RECOGNITION OF NON-SELF MOLECULES BY THE ADAPTIVE IMMUNE SYSTEM

The adaptive immune system of lymphocytes faces the same problem as the innate immune system, namely how to distinguish between self and non-self molecules. However the strategy employed by adaptive immune system is completely different from those which evolved for the innate immune system. Adaptive immune recognition is mediated by two types of antigen receptors: T cell receptor (TCR) and B cell receptor (BCR). These receptors are highly specific, and constitute a vast recognition repertoire that ensures that the adaptive immune system can detect any possible antigen encountered throughout life (1-3). TCR and BCR diversity is generated through site-specific DNA recombination and each receptor of a particular specificity is expressed in a unique clone of lymphocytes.

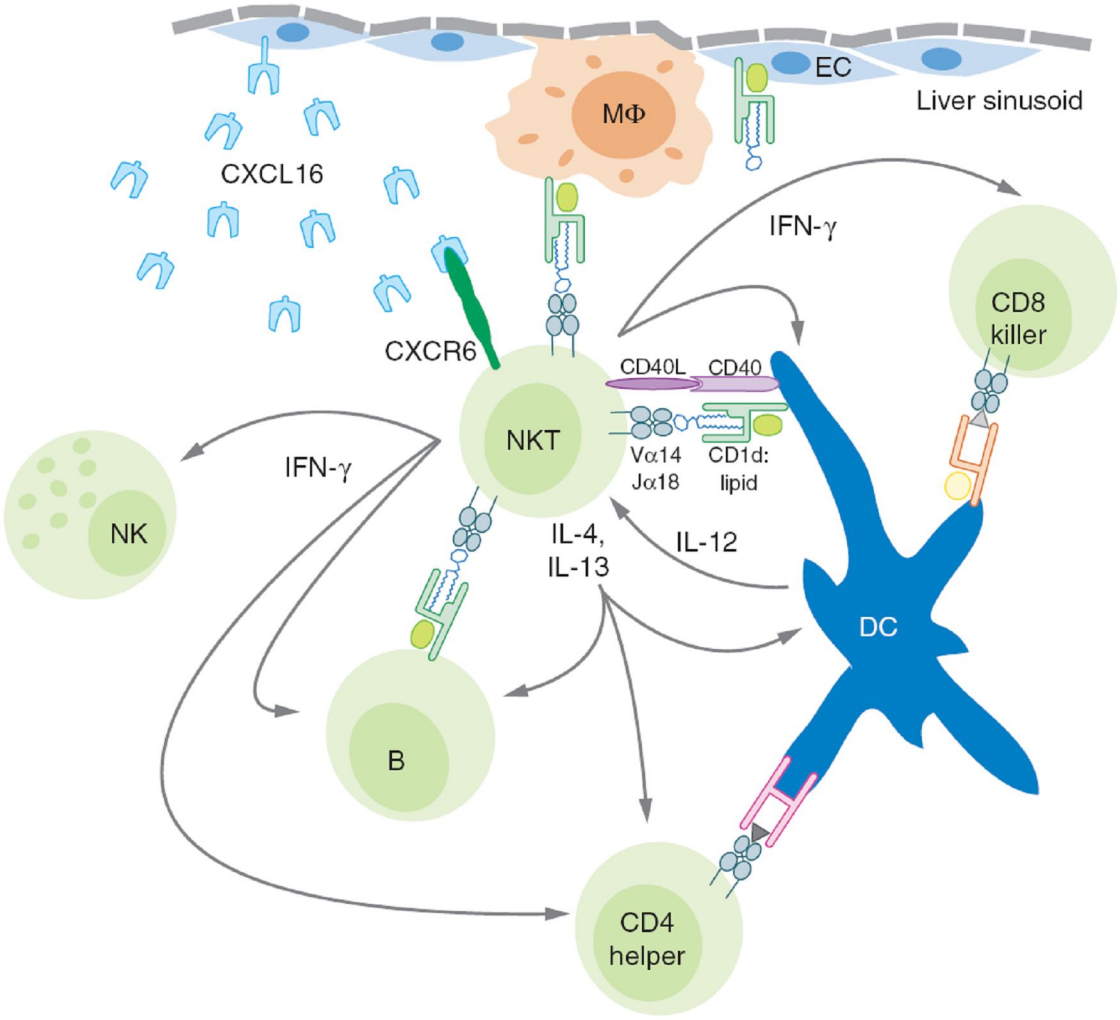


Figure 1.7 Cellular and molecular networks of NKT cells (72)

Due to the tremendous repertoire of specificities of such receptors also binding specificities towards self-molecules are found amongst them. To avoid self-reactivity, lymphocytes that react with self antigens are deleted during lymphocyte development (clonal deletion). This process is called induction of self-tolerance (1).

Importantly, T cells are peculiar in their way to recognize their cognate antigen. The TCR can recognize antigens in a form of "denaturated, unfolded, sequential determinants" and only when they are bound to MHC molecules (antigen presentation). $CD4^+$ T cells recognize antigens presented by MHC II and $CD8^+$ T cells recognize antigens presented by MHC I. In contrast, B cells can recognize free antigen without prior modification. In both cases, recognition of cognate antigen initiates the

proliferation of specific lymphocyte clones and expands the clone to a number sufficient to be effective in the immune defense reactions (clonal expansion).

In addition to direct activation of innate immune defense PRR also participate in the induction of adaptive immune recognition. Once a lymphocyte recognizes the antigen by its specific receptor, it requires instruction about the origin of the antigen. Such instructions are often derived from signals that are induced by PRRs on so-called antigen presenting cells (APCs). This is one of the basic principles of innate control of adaptive immunity, i.e. antigen recognized by lymphocytes associate with signals derived from recognition of PAMPs by PRRs (1-3, 76).

Which cells can activate T cells *via* antigen presentation and how antigen is processed and presented is described in details below.

ANTIGEN PROCESSING AND PRESENTATION

Since the discovery that T cell mediated immunity relies on "denaturated, unfolded, sequential determinants" of proteins immunologists have been fascinated with antigen processing and presentation. Many years of work have elucidated the pathways that generate peptide-MHC complexes. Now most of the components and mechanisms of the two major pathways of antigen processing and presentation are known (1, 77).

1. ANTIGEN PRESENTATION *VIA* MHC I

The MHC I presentation pathway is active in virtually all nucleated cells. It provides a mechanism for displaying at the cell surface a sample of peptides derived from proteins that are synthesized within the cell. This is true for self-proteins in steady state as well as for instance for viral proteins of infected cells. In consequence, the internal content of the cell is constantly displayed for detection by cytotoxic $CD8^+$ T cells allowing them to efficiently recognize and kill infected targets.

1.1 STRUCTURE OF MHC I

The structure of the MHC I molecule is optimized for presentation of particular antigens. The three-dimensional structure of MHC I was resolved by P.J.Bjorkmann in

1987 (78). Classical MHC Ia molecules are trimeric structures comprising a membrane bound heavy chain with three extracellular domains (α1, α2, α3) that associates non-covalently with β_2-microglobulin (β_2m) and a small peptide from 8-10 amino acids in length (Fig. 1.8). The α1 and α2 are highly polymorphic domains. The two helices and the 8 β-pleated sheets of α1 and α2 form a groove for peptide-binding and presentation. The conserved immunoglobulin-like α3 domain is found proximal to the membrane and is connected with the transmembrane domain followed by a short inatracellular cytoplasmic tail (1, 79-81). In addition to classical MHC I molecules there are non-classical MHC Ib molecules, like the CD1d molecule with a binding groove for lipid antigens, which will be described further below.

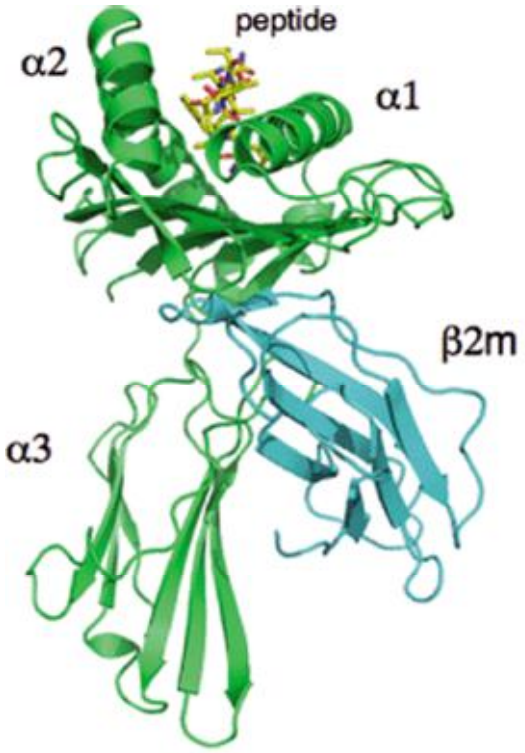


Figure 1.8 Ribbon representation of MHC I molecule with bound peptide (1)

MHC I molecules are assembled in the lumen of the endoplasmic reticulum (ER). It requires help of molecular chaperones such as calreticulin, ERp57 and the accessory molecule tapasin (1, 79-81). The α1 and α2 domains of MHC I molecules are very variable due to high genetic polymorphism. In fact, MHC molecules represent the most polymorphic genes of mammals. Three independent loci are found to encode different MHC Ia molecules in both human and mouse, although many mouse strains

carry only two of such MHC I genes. Therefore each heterozygous individual can express up to six different MHC I molecules. The protein encoded by each allele can bind different sets of peptides. The total number of distinct peptides that can bind to a MHC I molecule of a given allele is estimated in the billions (1, 79-81). This might be essential for the immune system to fight fast proliferating pathogens that otherwise might escape immune recognition.

1.2 PROCESSING OF ANTIGENS FOR MHC I PRESENTATION

Peptides presented on MHC I molecules are derived from intracellular antigens (both pathogen encoded and host derived). They are generated primarily by the ubiquitin-proteasome pathway in the cytosol. Presentation *via* MHC I favours newly synthesized proteins and especially aberrant translation products (defective ribosomal products - DRiPs) for degradation. Therefore, such proteins are captured before they transit to various other locations within the cell, which would be otherwise distant from the antigen presentation machinery. This mechanism allows also presentation of proteins with long half-lives and newly translated viral gene products early during infection (1, 80, 82).

1.2.1 Degradation of antigens by proteasome

Recognition of proteins by the degradation machinery for MHC I presentation usually requires ubiquitinylation. This multienzymatic step was shown to be dependent on several chaperones, like Hsp90 (1, 80, 81). Such ubiquitinated proteins are then targeted to the proteasome for proteolysis.

Two types of proteasomes exist: 26S proteasome, which is active under non-inflammatory conditions and the so-called immunoproteasome, which is assembled after exposure of target cells to IFN-γ. The 26S proteasome is composed of a 20S core particle and a 19S regulatory complex. The 20S core part is responsible for substrate proteolysis, while 19S part is responsible for substrate recognition.

The 26S proteasome exhibits three enzymatic activities: chymotrypsin like (cleavage after hydrophobic residues), trypsin like (cleavage after basic residues), and caspase like (cleavage after acid residues). Stimulation with IFN-γ leads to incorporation of

new catalytic subunits into the proteasome and the immunoproteasome is generated (Fig. 1.9). In general the proteasome produces peptides 2-25 amino acids in length, most of which are rapidly degraded by cytoplasmic peptidases (79, 80, 83).

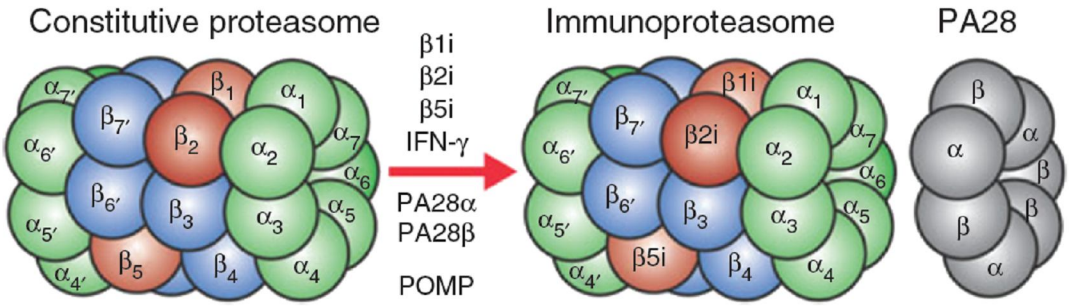


Figure 1.9 Formation of immunoproteasome (84)

1.2.2 Proteasome independent antigen processing

Most of the peptides for MHC I presentation are generated in a proteasome dependent way. Nevertheless, the use of specific inhibitors for proteasomes revealed that generation of some peptides could not be blocked. This suggested that alternative mechanisms have to exist. One potential candidate appears to be tripeptidyl peptidase II (TPPII). Degradation by TPPII is however limited to few proteins only. In most cases TPPII acts as a downstream of the 26S proteasome. Hence, it cleaves products that are released from the 26S proteasome (80, 85).

1.2.3 Post-proteasomal processing of antigens

The proteasome destroys more peptides than it generates. Only 15% of peptides generated *via* proteasomal degradation have the correct size. The majority of them is too short to be presented. Presentable peptides generated by the proteasome have the correct C-terminus end to bind to the peptide binding groove of MHC I, most likely due to co-evolution of both complexes. However they might still require further trimming at the N-end. This can be achieved by aminopeptidases present in the cytosol

and the ER (80, 86, 87). To this group of enzymes belong leucine aminopeptidase and puromycin-sensitive aminopeptidase, which are present in the cytosol (86, 87).

1.2.4 Final trimming of peptides and loading on MHC I molecules in the endoplasmic reticulum

Peptides generated in the cytosol of the cell have to be translocated across the ER membrane in order to be loaded onto MHC I molecules. This transport can be achieved by the transporter associated with antigen processing (TAP) localized in the membrane of the ER and *cis*-Golgi (1). TAP is a heterodimer composed of two subunits, TAP1 and TAP2, both of which are essential and sufficient for peptide translocation. Peptides transported *via* TAP have a length between 7 to over 20 amino acids. Therefore some of them still require trimming before loading onto the MHC I molecule is complete. At least two aminopeptidases are known to be involved in this process: endoplasmatic reticulum associated peptidase I (ERAP I) and L-RAP. Trimmed peptides will then fit into the binding groove of the assembling MHC I molecules (1, 80, 83, 86).

MHC I/peptide assembly requires a number of chaperones. They help in folding of the MHC I heavy chain, and its binding to β_2m. Tapasin is the protein, which physically bridges the dimer of heavy chain and β_2m to TAP. Tapasin also allows only high affinity peptides to bind. Other chaperones involved in this process are calreticulin and thiol oxidoreductase ERp57. ERp57 is crucial for the recruitment of MHC I molecules into the peptide loading complex, calreticulin appears to be important for the optimization of peptide loading (1, 80, 83, 86). Once peptide is loaded onto MHC I, the complex is transported *via* the constitutive secretory pathway through the Golgi apparatus to the cell surface. Here the MHC I/peptide complex can be recognized by the TCR of $CD8^+$ T cells (Fig. 1.10, 1.11) (81, 83).

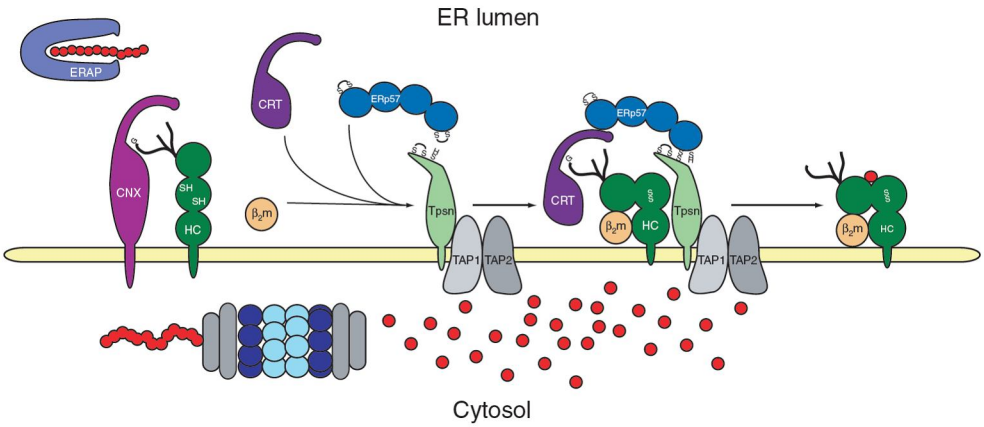


Figure 1.10 MHC I assembly and loading pathway (88)

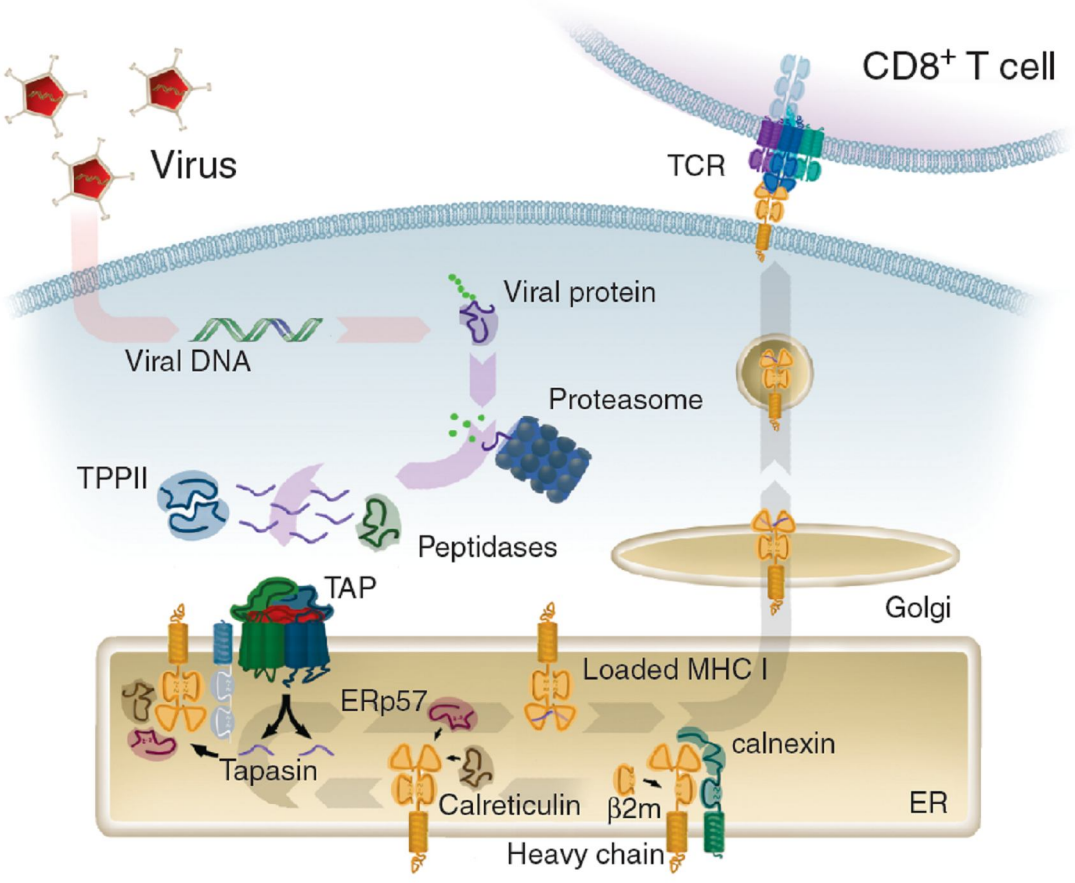


Figure 1.11 Classical pathway of MHC I antigen processing (89)

1.2.4 Cross-presentation pathway

Cross-presentation was initially observed by Michael Bevan in 1976 (90). He showed that priming of cytotoxic $CD8^+$ T cells can be initiated *via* presentation of exogenous antigens on MHC I molecules. He called it "cross-priming", therefore presentation of exogenous antigens *via* MHC I, which results in cross-priming of $CD8^+$ T cells is called cross presentation. Only DCs were shown to be able to cross-present exogenous antigens. The mechanism of cross-presentation allows DCs to induce protective immune response against pathogens which do not directly infect DCs or against tumors which do not originate from DCs (91, 92). Cross-presentation is generally biased toward cell-associated antigens and antigens released during cellular destruction but autoantigens and soluble antigens are also cross-presented but with lower efficiency (1, 91, 92). The cell-biological mechanisms underlying of cross-presentation are not fully resolved yet, but nearly complete scenario for soluble antigen came out in 2008 due to the work of Sven Burgdorf and Christian Kurts (91).
They showed that cross-presentation is spatially and mechanistically separated from classical presentation of endogenous antigens *via* MHC I. The scenario for cross-presentation of soluble antigen appears as follows: soluble protein is taken up by DCs into compartments specialized for cross-presentation, i.e. early endosomal compartments. From there, the protein is exported into the cytosol (the mechanism is not fully resolved yet, probably *via* sec61 molecules) and degraded by the proteasome. Products of degradation are reimported *via* TAP into the same endosomal compartment for loading onto MHC I molecules. From these endosomes, cross-presented antigen is transported directly to the cell membrane for presentation to $CD8^+$ T cells (Fig. 1.12). Importantly, here for the first time authors showed the presence of specialized early endosomal compartments, which most probably are a prerequisite for efficient cross-presentation (91). This experimentally established mechanism still raises a lot of questions, which hopefully can be answered in near future.
It is important to mention, that another group recently showed presence of antigen storage compartments, characterized as a lysosome-like organelle (93). This intracellular antigen depot facilitates prolonged cross-priming capacity of DCs, once immune complexes are antigen source. Nevertheless, whether this compartment could

cooperate during cross-presentation with early endosomal compartment showed by Burgdorf et.al.(91), is still very enigmatic.

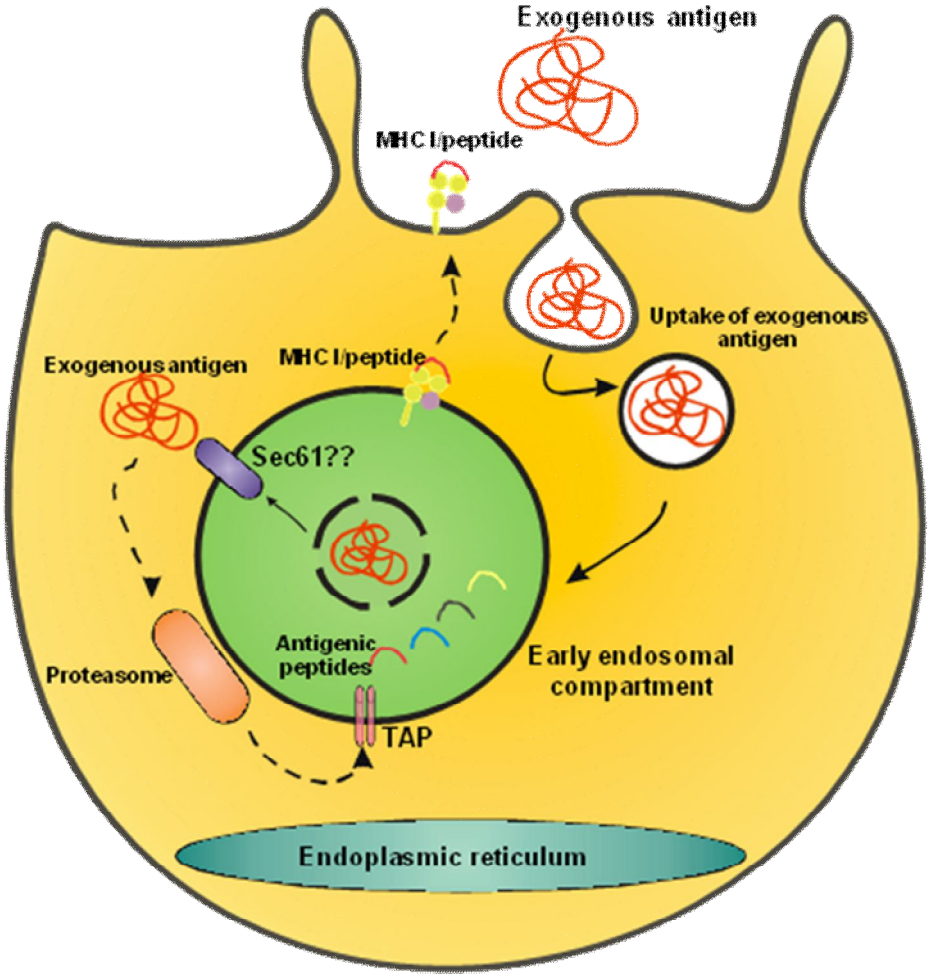


Figure 1.12 Cross-presentation pathway, adapted from (91)

2. ANTIGEN PRESENTATION *VIA* MHC II

The MHC II pathway, also called endocytic pathway, is restricted only to professional APCs which constitutively express MHC II molecules, like DCs and B cells. MHC II molecules bind peptides derived from antigens of exogenous origin that are internalized by endocytosis and are processed within the endocytic compartments. MHC II molecules can also present peptides derived from endogenous proteins in a process called autophagy. Autophagy is believed to deliver cytoplasmic proteins to

lysosomes (1, 83). Presentation of antigen *via* MHC II leads to activation of specific $CD4^+$ T cells which further may regulate almost all components of adaptive immunity (1, 83).

2.1 STRUCTURE OF MHC II

The X-ray structure of MHC II was determinated in 1993 by Jerry H.Brown (94). MHC II molecules contain two different polypeptide chains of nearly equal size - α and β chains. They are non-covalently associated together. Each chain contains two external domains: α1 and α2 domains and β1 and β2 respectively (Fig. 1.13). Structurally MHC II molecules show high similarity with MHC I molecule especially concerning the peptide binding groove, yet the binding groove of MHC II can bind longer peptides, 13-18 amino acids in length (1).

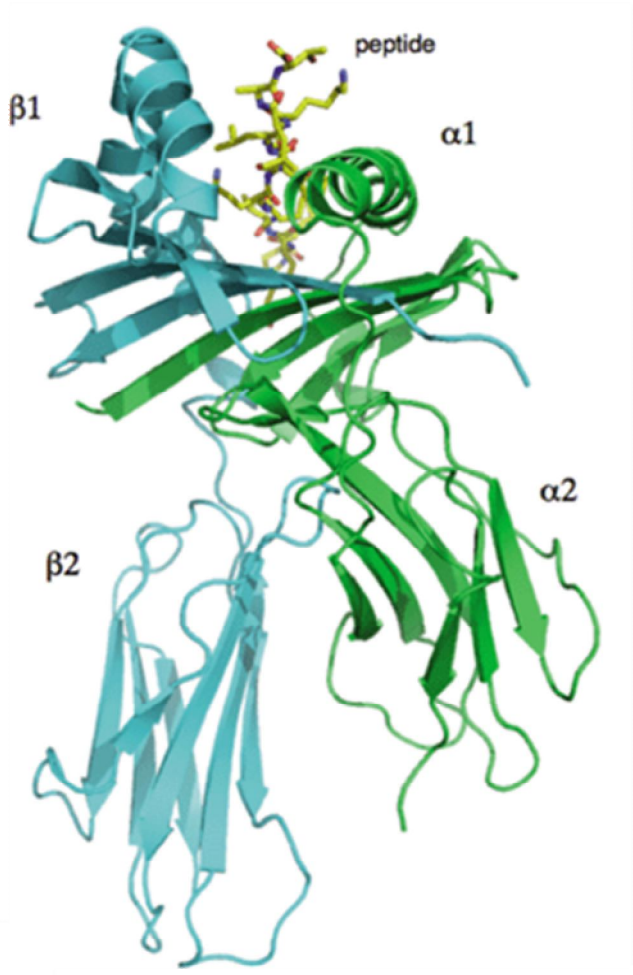


Figure 1.13 Ribbon representation of MHC II molecule with bound peptide (1)

Upon synthesis in the ER, the α and β chains immediately associate and assemble with a third protein, the invariant chain (Ii). The Ii serves both to prevent random peptides from binding to the groove of the MHC II molecule on their way to proper subcellular site, and to direct MHC II molecules to this cellular compartment. The destination site for empty MHC II molecules are late endosomal compartments called MHC II compartments (MIIC) (1, 83) (Fig. 1.14). Interestingly, recently Ii was also shown to regulate migration of DCs (95).

2.2 PROCESSING OF ANTIGENS FOR MHC II PRESENTATION

Peptides loaded onto MHC II molecules are generated after endocytosis by endosomal/lysosomal proteases. Enzymes involved in this process are: endopeptidases, exopeptidases and IFN-γ induced lysosomal thiol reductase (GILT). Most of these enzymes exhibit broad specificities and their action is most likely redundant. The activity of such enzymes is regulated by several factors including pH (acidic pH), concentration of endogenous inhibitors, activators and chaperones (1, 87, 96)

2.2.1 Processing of Ii and peptide loading onto MHC II molecules

Once the MHC II/Ii complex is assembled in the ER and has reached the MIIC removal of Ii is started. Degradation of Ii is mediated by cysteine proteases like cathepsin S and L. This process leads to the formation of the peptide - CLIP (class II invariant chain-associated peptide), which still occupies the MHC II binding groove. CLIP has to be exchanged for an antigenic peptide to from a proper MHC II/peptide complex. This exchange is facilitated by the chaperon H2-M, which directly interacts with MHC II molecules and stabilizes its open conformation. Antigenic peptides for loading onto the binding groove of MHC II molecules are available due to fusion of endo-lysosomal processing compartments with the MIIC. This allows the exchange of CLIP with antigenic peptides. However, only high affinity peptides form MHC II/peptide complexes with a closed loaded conformation for a sufficient length of time for the MHC molecules to be released from H2-M and to be transported to the cell surface (kinetic proof reading). Such MHC II/peptide complexes can be stable for long

time at the cell surface and can be recognized by the TCR of $CD4^+$ T cells (Fig. 1.15) (1, 83, 87, 96).

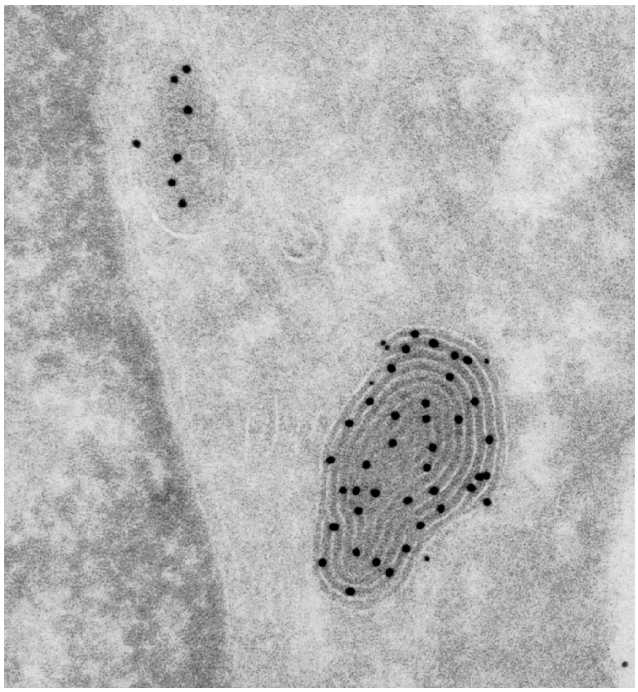


Figure 1.14 Electron microscopy micrograph of ultrathin cryosection of DC. Immunogold labeling of CD1b (small gold) and MHC II (large gold) within MIIC (173).

3. PRESENTATION OF LIPID ANTIGENS *VIA* CD1

CD1 molecules are MHC-like proteins (MHC Ib) that bind and present lipid antigens. Still little is known about intracellular pathway leading to lipid presentation. The CD1 family consists of five members (CD1a to e) with multiple potential isoforms of each gene. Humans carry all five genes whereas mice posses only the CD1d gene. These molecules present self and non-self lipid antigens to a specialized population of T cells, NKT cells (1, 97-100).

3.1 STRUCTURE AND TRAFFICKING OF CD1d MOLECULES

CD1d is a type I integral membrane protein with the α1, α2, α3 extracellular domains of heavy chain non-covalently associated with $\beta_2$microglobulin (β_2m), analogous to MHC I molecule. A major difference is that CD1 molecule has a more limited

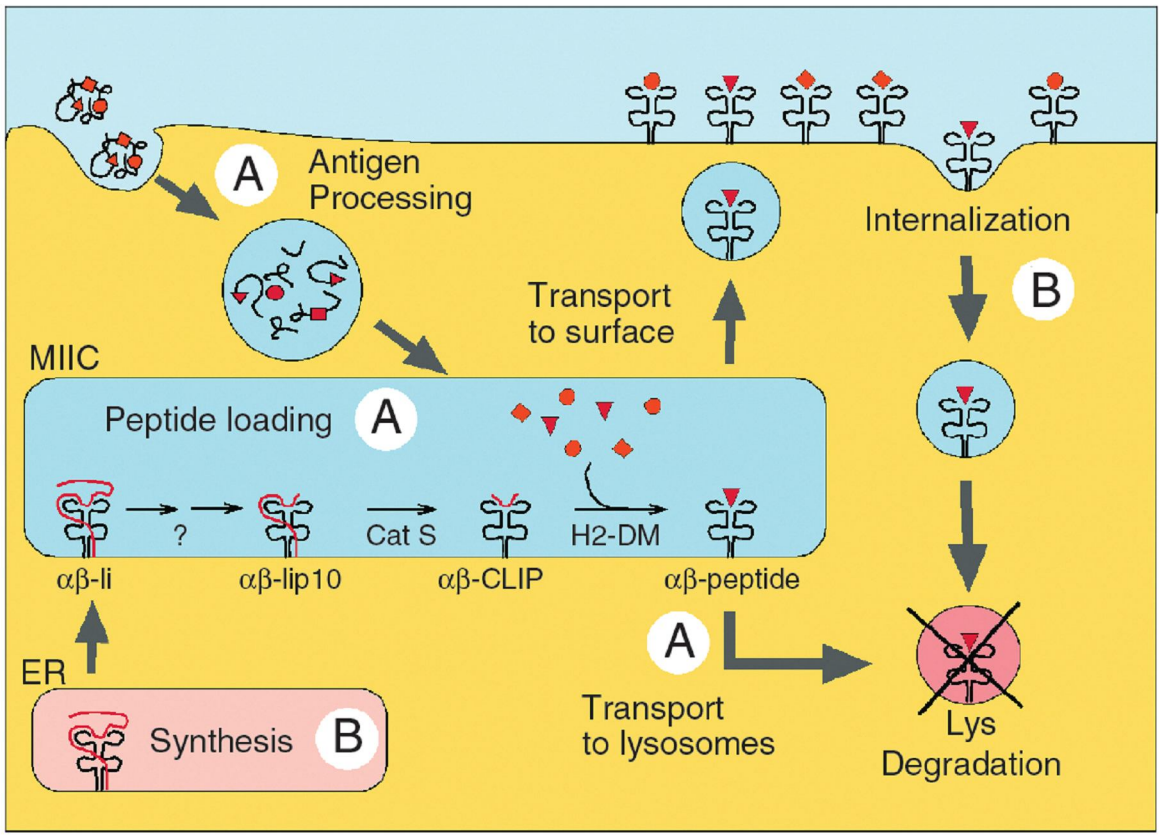


Figure 1.15 MHC II presentation pathway (32)

polymorphism in comparison to MHC I molecules. The structure of CD1d is quite flexible and unstable when not filled with ligand (Fig. 1.16) (1, 100).

Newly generated CD1 α chains are glycosylated in ER and correct folding is assisted by interaction with several chaperones like calnexin, calreticulin and ERp57. Further stabilization of the complex is followed by association with β_2m. However, the existence of β_2m free forms of CD1d has been also reported. Interestingly, these forms were also able to activate CD1-restricted T cells (57, 60). The antigen binding groove of the CD1 molecule is relatively large and hydrophobic. In the ER newly synthesized CD1 molecules are loaded with endogenous lipids to stabilize the antigen binding groove. To such lipids belong: glycosylphosphatidylinositol (GPI) and phosphatidylinositol (PI). The loading of lipid antigens requires activity of lipid transfer proteins like microsomal triglyceride transfer protein (MTP) (97-99).

Subsequently, CD1 molecules migrate through the Golgi apparatus to the cell surface. From here they are re-cycled by entering the so-called sorting endosomal compartments with help of the AP-2 adaptor protein. In the next step, CD1 molecules

interact with the AP-3 adaptor protein and are directed deeper towards lysosomes and the MHC II compartments (MIIC) (97-99).

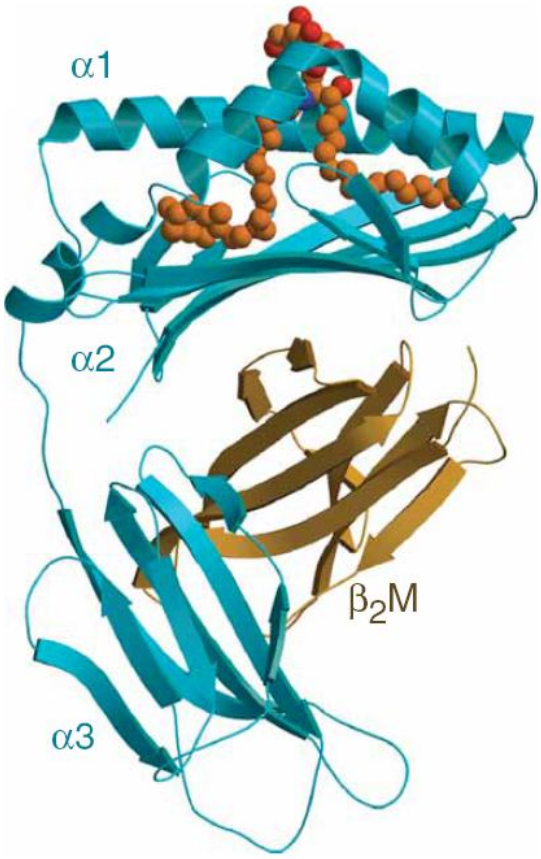


Figure 1.16 Ribbon representation of human CD1d with αGalCer (101)

3.2 PROCESSING AND PRESENTATION OF LIPIDS

Lipid antigen uptake occurs *via* endocytosis. Several proteins are known to mediate engulfment of such lipid and glycolipid antigens, like apolipoprotein E (Apo-E). Apo-E can efficiently deliver lipid antigens to endosomal compartments, where they are processed by different endosomal/lysosomal glycosidases. Next lysosomal lipid transfer proteins, such as saposins might transfer processed lipid antigens for loading onto CD1 molecules. Loading of lipid antigens onto CD1d molecules is enhanced at acidic pH. The CD1/lipid antigen complexes are delivered to the cell surface for presentation to CD1-restricted T cells (Fig. 1.17) (97-99).

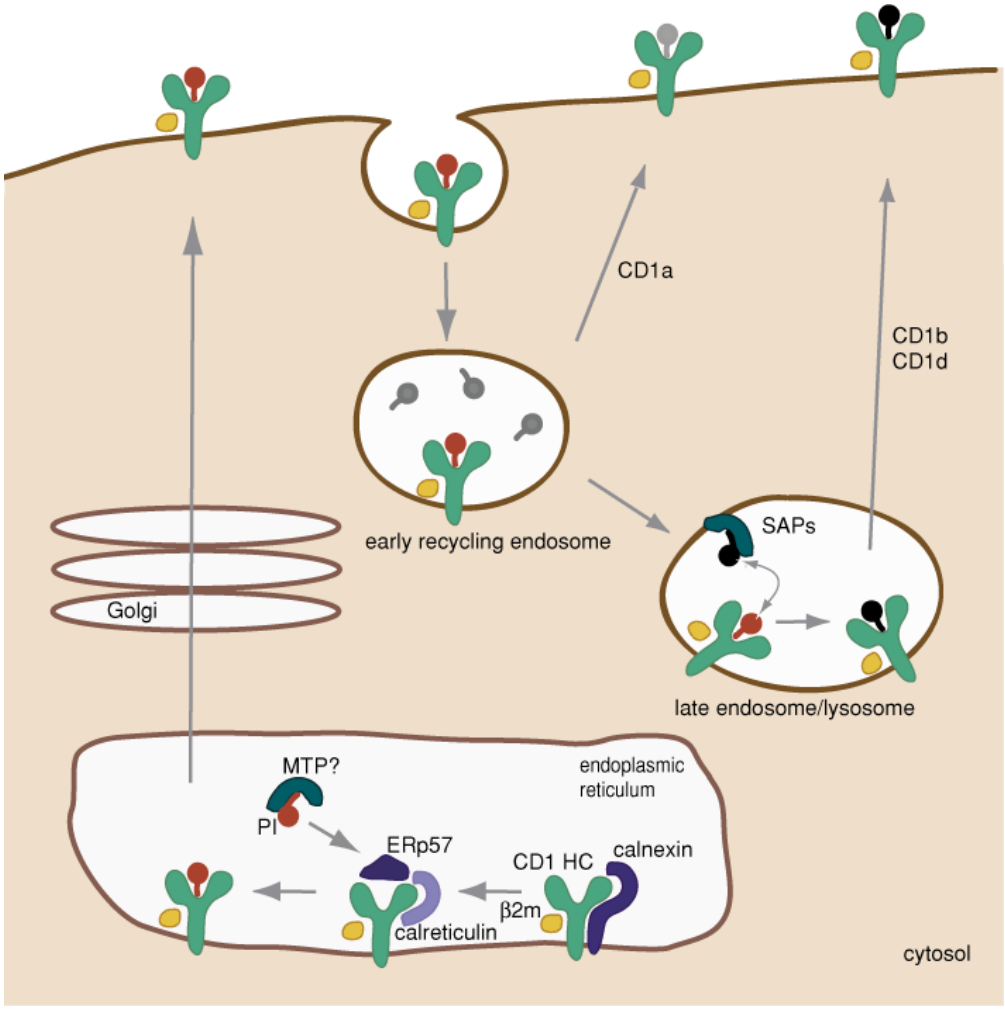


Figure 1.17 CD1d presentation pathway (1)

4. HEAT SHOCK PROTEINS IN ANTIGEN PRESENTATION

Interestingly, heat shock proteins (Hsp) have been shown to be involved in antigen processing and presentation. For instance, as mentioned above, Hsp90 is required for the ubiquitination of proteins destined for processing. But other activities were also observed.

Heat shock proteins were discovered in 1974 (102). They are categorized into several families and named on the basis of their approximate molecular weight (103). These proteins are localized in various cellular compartments and may function as molecular chaperones. They can take part in the assembly, stabilization, folding and translocation of proteins and peptides (103, 104).

Hsp70 is the most conserved protein in evolution. Chaperoning function for Hsp70 proteins is characteristic and essential for Hsp70-mediated protection against stress, but recently new house-keeping roles of constitutively expressed Hsp70 proteins were discovered. This includes transport of proteins between different cellular compartments, degradation of unstable proteins, folding and refolding of proteins (103, 104). In addition to this very generalized role for Hsp70, they have been shown to regulate immune response *via* promoting of antigen processing and presentation (105-108) Hsp70 proteins are involved in chaperoning proteins/peptides during degradation and during antigen presentation *via* both MHC I and MHC II.

In the antigen presentation *via* MHC I molecules Hsp70 protein was shown to physically associated with TAP, hence enabling efficient loading of chaperoned peptides onto MHC I molecules in the ER (105, 106, 109). Moreover, Hsp70 was shown to stabilize peptides after proteasomal degradation on their way to the ER (103). In the context of cross-presentation peptides chaperoned by Hsp were shown to be a necessary and sufficient source of antigen for efficient cross-priming of $CD8^+$ T cells (106, 110).

The importance of Hsp70 during MHC II presentation pathway was also clearly demonstrated (105, 111), however exact mechanism and location of its action is still under debate. One possibility is that Hsp70 assist in loading of peptides on MHC II in the MIIC. However it is not clear how soluble Hsp70 molecules access the vesicular MIIC compartments. Another possible role of these proteins in MHC II presentation is their help to deliver endogenous proteins during autophagy into lysosomes *via* LAMP-2 molecules (Fig. 1.18) (112). Yet, this mechanism still remains to be elucidated.

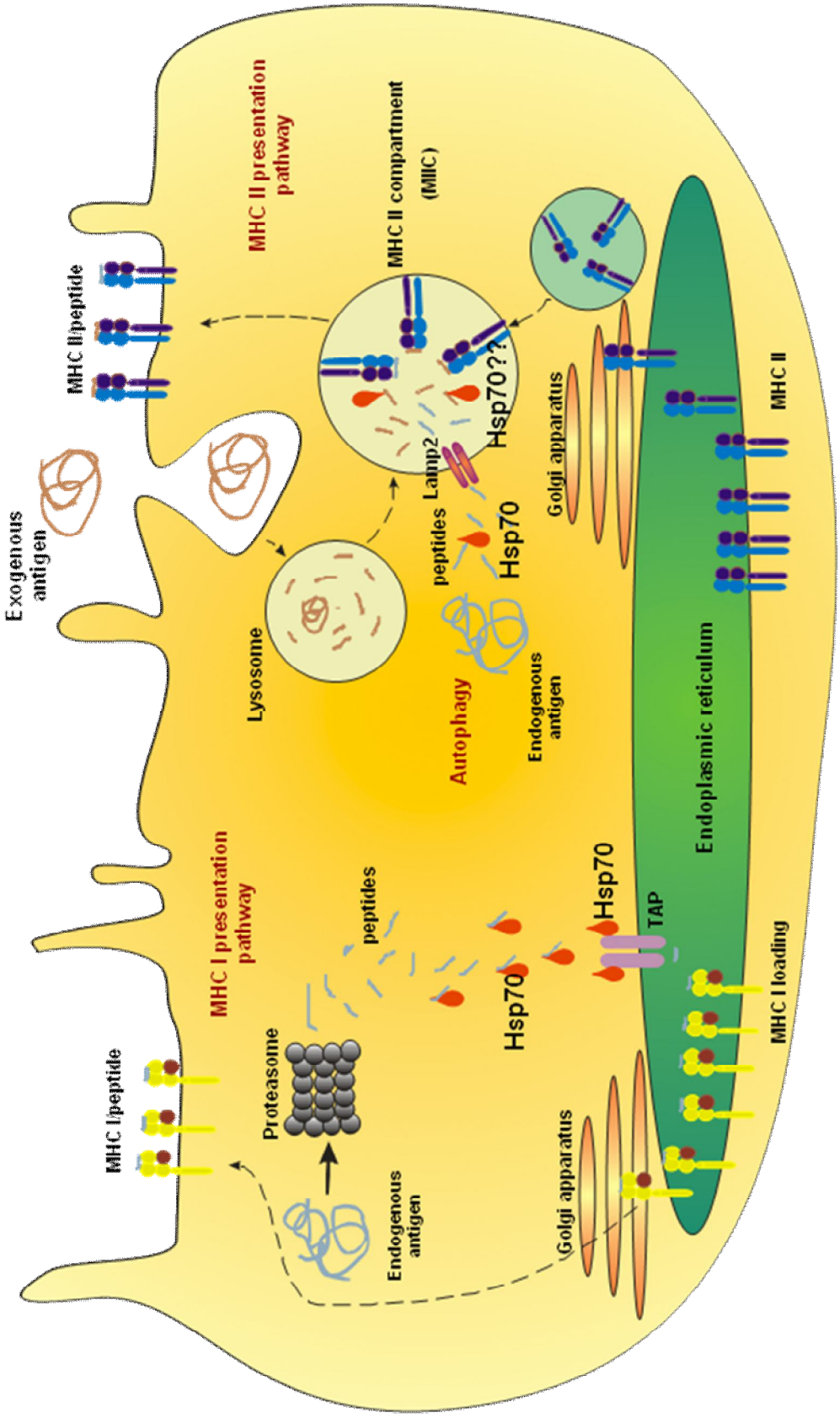


Figure 1.18 Heat shock proteins in antigen presentation, adapted from (105, 109, 111)

5. CO-STIMULATORY MOLECULES

Interaction between MHC/peptide/TCR only is not sufficient for efficient clonal activation and expansion of resting naive T cells. For activation and acquisition of proper effector functions T cells require a second type of signal which is called co-stimulation. Co-stimulatory molecules are expressed on surface of APCs and this signaling ensures, that T cells will respond properly to triggering of their TCR (113-115). Co-stimulatory signals may exhibit both activating and inhibitory effect on T cells. Proper balance between those two opposite activities is necessary for induction of specific immune response as well as to maintain tolerance and prevent autoimmunity. Signals derived *via* co-stimulatory molecules are particularly important for naive T cells but they also play a role during activation of antigen experienced T cells (113-115). Below the most relevant co-stimulatory pathways for this work are described.

CD80/CD86 – CD28/CD152 pathway is the best characterized T cell co-stimulatory pathway. Two members of the immunoglobulin-like B7 family CD80 (B7-1) and CD86 (B7-2) are expressed on cell surface of APCs and interact with the T cell associated co-receptors CD28 or CD152 (CTLA-4). This pathway is crucial for T cell activation as well as for maintenance of immunological tolerance. CD80 and CD86 have highly overlapping functions, whereas CD28 on T cells transmits activating signals and CD152 delivers inhibitory signals (113-115).

ICOS-ICOSL pathway appears to be particularly important for stimulating effector T cell responses and T cell dependent B cell responses, as well as in regulating T cell tolerance. ICOS (a member of the B7 family) has a unique function to regulate $CD4^+$ T cell differentiation. ICOS expressing T cells were shown to secrete T_H2 cytokines (IL-10, IL-4) (115) and ICOS-ICOSL signaling plays an important role in B cell differentiation, immunoglobulin class switching, germinal center formation and memory B cell development (115). ICOSL can be expressed on endothelial and epithelial cell as well as professional APCs (113-115).

At the moment the B7 family of co-stimulatory molecules is still growing. In addition new functions for old family members are discovered (115).

CD40-CD40L pathway is crucial for induction effective T cell and B cell responses, additionally it facilitates the final maturation of APCs. CD40 and CD40L (CD154) molecules belong to the TNF and TNF receptor superfamily. Once $CD4^+$ T cell will recognize antigen, they will rapidly up-regulate the expression of CD40L. Engagement of CD40 on the surface of APCs leads to production of IL-12 which is a potent inducer of T_H1 immune response. Moreover, CD40-CD40L interaction leads to up-regulation of other co-stimulatory molecules, like CD80 and CD86. As consequence, APCs become more mature and can more efficiently prime T cells (1).

Thus it becomes clear that a highly complex interaction involving a multitude of molecules, pathways and cell types is required to induce a proper and effective immune reaction that still requires major efforts to be fully understood.

AIM OF THE WORK

AIM OF THE WORK

Despite the multitude of potential pathogens that we constantly encounter, only very few are able to cause severe disease. The main reason for this is that our immune system is able in almost all cases to respond in an appropriate way. In these reactions T cells play a major role. However, T cells are not able to recognize a pathogen directly. Only processed fragments of pathogens are seen by the T cells. Thus, T cells need to be instructed by professional antigen presenting cells (APCs) like DCs, B cells or macrophages on the nature of the antigen i.e. they have to be imprinted to carry out the most appropriate effector function. To understand mechanisms underlying the initiation of such T cells responses many factors need to be consider. Thus, the environmental conditions where APCs develop and mature, their strategical location and cross-talk with other immune cells might play a major role as well as the overall systemic conditions of the organism like steady state conditions or inflammation.

The aim of presented here work was therefore to investigate the influence of different developmental and environmental situations on the ability of splenic conventional DCs (cDCs) and MZ B cells to present antigen and induce T cell activation in steady state conditions. In details:

- Development, migration and maturation of DCs are strongly influenced by cytokines present in their environment. One class of such cytokines are type I IFNs. Since the role of type I IFNs during viral and bacterial infections as well as their influence on immune cells are well studied, I decided to investigate how constitutive levels of type I IFNs regulate cell mediated immunity. A few studies had shown spontaneous low level production of type I IFNs. It was proposed that these constitutive levels maintain cells in "primed" state enabling them rapid and robust response after encounter of a pathogenic stimulus. How these constitutive levels of type I IFNs might influence antigen presenting cells and the induction of adaptive immune responses was not known. Therefore in the present work I intended to characterize splenic cDCs from IFN-β or IFNAR deficient mice and to test them for antigen presentation and T cell stimulatory capacity. Additionally, I decided to establish the transcriptional program of such DCs by microarrays and possible conjunction with other proteins that are involved in antigen presentation.

- Development and function of splenic DCs are also influenced by the presence of other immune cells. Few studies showed that the presence of T and B cells is necessary for proper maturation of DCs and their ability to present antigen and stimulate T cells. Nevertheless, the reasons for this influence were so far not investigated in details. Hence, I wanted to characterize splenic cDCs from $RAG^{-/-}$ mice deficient in T and B cells and test them for their ability to present soluble and cell associated antigens. The analysis should include the transcriptional program of such DCs and how their lacking functions could be recovered.

- Particular anatomical location of APCs, their access to antigens and interactions with effector cells can strongly influence the way and character of antigen presentation and therefore T cell differentiation. One example of a strategical location is the marginal zone (MZ) in the spleen and MZ B cells in particular. Detailed characterization of such cells revealed that they express the highest levels of the lipid presenting molecule CD1d among all splenic APCs. Thus, MZ B cells may play an important role in activation of invariant NKT cells, an effector cell type that is able to rapidly respond to antigen challenge. Therefore, I intended to test MZ B cells for their ability to present lipid antigens. The consequence of this presentation on differentiation of NKT cells in comparison to presentation by cDCs should also be investigated.

This work should provide new insights first of all into how spontaneously produced type I IFNs ensures that DCs properly present antigens during steady state conditions. This could potentially influence maintenance of peripheral tolerance to self antigens. Then it should shed light on how character and specialization of DCs is imprinted by the presence of T and B cells during development and differentiation. Finally, it should provide clues how APCs of a particular anatomical location that has immediate access to antigen can dictate the outcome and character of immune response.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

2.1 Mice

Female wild type (WT) C57BL/6 mice were purchased from Harlan-Winkelmann (Borchem, Germany and AN Venvay, The Netherlands).

Female IFN-$\beta^{-/-}$(116), IFN-$\beta^{+/+}$ controls, IFNAR$^{-/-}$ (117) C57BL/6 mice, OT I (transgenic for CD8^{+} T cells expressing a TCR specific to $OVA_{257-264}$ peptide) and OT II (transgenic for CD4^{+} T cells expressing a TCR to $OVA_{323-339}$ peptide) mice (118, 119), RAG1$^{-/-}$ (120) and RAG2$^{-/-}$ C57BL/6 (121) mice were bred at the animal facility of the Helmholtz Centre for Infection Research (HZI).

The initial generation of Hsp70.1/3 knockout mice has been previously described (122). The C57BL/6 Hsp70.1/3$^{-/-}$ mice were backcrossed from a 129 background onto the C57BL/6 background and were raised at Washington University School of Medicine in St. Louis (kindly provided by Dr. Clayton Hunt and Prof. Tej K. Pandita).

The Vα14 transgenic mice (123), C57BL/6 CD1d$^{-/-}$ (124) and C57BL/6 $\beta_2$microglobulin$^{-/-}$ were kindly provided by Dr. Florian Winau (Max-Planck-Institute for Infection Biology, Berlin) and by Prof. H. Robson MacDonald (Ludwig Institute for Cancer Research, Lausanne, Switzerland).

All mice were used between 8 to 12 weeks of age. Mice were bred and maintained in specific pathogen free conditions. Mouse care and experimental procedures were performed under approval of the local authority LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit)

2.2 Cell lines

The B3Z T cell hybridoma (125) specific for the H-2·K^{b}/SIINFEKL complex was maintained in IMDM supplemented with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin, 50µM 2-mercaptoethanol, 2mM L-glutamine (complete IMDM). The hybridoma 25-D1.16 secreting an IgG1κ monoclonal antibody (mAb) specific for the pOV8·H-2K^{b} (126) was kindly provided by Dr Ronald Germain (NIH NIAID, Bethesda, USA). Antibody (Ab) was purified and conjugated with FITC according to

standard procedures. EG7-OVA cell line (127) (EL4 thymoma stably transfected with OVA cDNA, kindly provided by Prof. Michael Bevan, Scripps Clinics and Research Foundation, San Diego, USA) and EL4 cell line were maintained in complete IMDM.

2.3 Antibodies and Fluorescence-Activated Cell Sorting (FACS)

The following antibodies were used for flow cytometry:

SPECIFICITY	CLONE	FLUOROCHROME	COMPANY
CD16/CD32	2.4G2	none	BD Pharmingen
CD11c	N418	APC or PE-Cy7	eBioscience
CD11b	M1/70	PE-Cy7 or Pacific Blue	eBioscience
B220 (CD45R)	RA3-6B2	APC-AlexaFluor 750 or APC-Cy7	eBioscience or BD Pharmingen
CD4	GK1.5	PE	eBioscience
CD8α	53-6.7	Pacific Blue, FITC or PE	eBioscience
H-2K^b	Y-3	FITC	Self purified and conjugated
I-A^b	M5/114.15.2	FITC	eBioscience
CD1d	1B1	PE	eBioscience
NK1.1	PK136	FITC, PE-Cy7 or PerCP-Cy5.5	Self purified and conjugated, eBioscience,
CD49b	Dx5	PE-Cy7 or PE	eBioscience
CD19	1D3	APC	BD Pharmingen
CD21/35	7G6	FITC or biotin	BD Pharmingen, self purified and conjugated
CD5	53-7.3	FITC, PE	BD Pharmingen,

CD23	B3B4	PE or biotin	eBioscience
CD80	16-10A1	PE	eBioscience
CD86	GL-1	FITC	eBioscience
CD28	37.51	APC	eBioscience
CTLA-4	UC10-4B9	PE	eBioscience
CD40	HM40-3	FITC	eBioscience
CD40L	MR1	PE	eBioscience
ICAM-1	YN1/1.7.4	PE	eBioscience
ICOS	7E.17G9	none or PE-Cy5	eBioscience
ICOSL	HK5.3	none or PE	eBioscience
GITR	DTA-1	none or PE	eBioscience
GITRL	eBioYGL386	biotin	eBioscience
B7-H1	MIH5	PE	eBioscience
B7-H4	eBioMIH29	PE	eBioscience
B7-DC	TY25	biotin	eBioscience
PD-1	RMP1	PE	eBioscience
OX40	OX-86	PE	eBioscience
OX40L	RMI134L	biotin	BD Pharmingen
CD27	LG.7F9	biotin	eBioscience
CD70	FR70	PE	eBioscience
CD48	HM48-1	FITC	eBioscience
CD244.2	eBio244F4	PE	eBioscience
H2-K^{b}/SIINFEKL	25-D1.16	FITC or APC	Self purified and conjugated or eBioscience
IL-4	11B11	PE	BD Pharmingen
IFN-γ	XMG1.2	Pacific Blue	eBioscience
IL-17	17B7	AlexaFluor 647	eBioscience

IL-13	eBio13A	AlexaFluor 647	eBioscience
Mouse IgG	Isotype control	none	Dianova
Mouse IgM, *κ*	Isotype control	none	BD Pharmingen
IL-12	C17.8	none	eBioscience

Flow cytometry analysis was performed using FACSCanto, LSRII (BD Bioscience), sorting was performed using FACSAria I and FACSAria II (BD Bioscience). The data were analyzed using FACSDiva (BD Bioscience) software, version 6.1.

2.4 Preparation of cell associated ovalbumin (OVA)

EG7-OVA and EL4 (control, non-transfected cells) cells were harvested from culture bottles and counted. Further 1×10^6 cell were UV irradiated, using an UV Crosslinker, with 9mJ/cm^2. Next, cells were washed intensively with PBS and used in antigen presentation assay (described in "Methods").

2.5 Biotinylation of OVA

Soluble OVA (EndoGrade OVA, Profos) (2mg/ml) dissolved in 0.1M $NaHCO_3$ buffer, pH 8.2, was incubated with 50µg of biotin ester for 1h with continuous stirring, followed by dialysis against PBS.

METHODS

2.6 Isolation of splenocytes

Spleen cells were prepared by gentle flushing out the spleens with complete IMDM. Erythrocytes were lysed for 2 min in ACK buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA) and washed two or three times in PBS. Cell clumps were removed by passage through a 50µm nylon filter. Splenocyte preparation was carried out strictly on ice. Cells were further used for staining with different Abs, FACS analysis or FACS sorting.

2.7 Staining of splenocytes and cell sorting strategy

Single cell suspensions were treated with anti-mouse CD16/CD32 BD Fc Block for 10 min followed by staining with appropriate Abs for 20 min on ice. Further cells were washed with PBS.

Splenic cDCs were sorted as cells with high side scatter ($SSC^{hi\ (high)}$) that were:

$CD11c^{hi}$, $CD8\alpha^{+/-}$, $CD11b^{+/-}$, $B220^{-}$

All B cells were gated on CD19 and were positive for following markers:

$CD21/35^{hi}$, $CD23^{-/lo(low)}$ (MZ B cells)

$CD21/35^{lo}$, $CD23^{+}$, $CD5^{lo}$ (B1 B cells)

$CD21/35^{lo}$, $CD23^{+}$, $CD5^{-}$ (B2 B cells)

Invariant NKT cells first were enriched by magnetic depletion of B cells and CD8 positive cells (Invitrogen). Next, cells were stained and sorted as follows:

NKT cells were negative for lineage markers (lin^{-}),

like $CD8\alpha^{-}$, $B220^{-}$, $CD11c^{-}$, $CD49b^{-}$, $CD11b^{-}$ (prepared as a lin^{-} coctail)

and positive for $NK1.1^{+}$, $CD4^{+}$, $TCR\alpha/\beta^{+}$, $CD3^{+}$

Final purity of all APCs was always >97%. Final purity of NKT cells was always above 99% and did not contain any $CD11c^{hi}$ cells. All samples during the sorting procedure were kept at 4°C.

2.8 Preparation of T cells

OT I (OVA specific $CD8^+$ T cells) and OT II (OVA specific $CD4^+$ T cells) were isolated from lymph nodes (subcutaneous and mesenteric) and spleen. Single cell suspensions were further purified using the CD8 or CD4 negative isolation kits (Dynal) containing Abs against B220, CD11b, Ter-119, CD16/32 and CD4 (for OT I isolation) or CD8 (for OT II isolation) following the protocol provided by the manufacturer. Cell preparations contained more than 90% of the desired cell population and were essentially free of $CD11c^{hi}$ cells as determined by flow cytometry using Abs specific for CD4 or CD8 and CD11c, respectively. For antigen presentation assays OT I or OT II cells were stained with 1µM CFSE (Molecular Probes) for 10 minutes at 37°C according to the manufacturer's protocol.

2.9 Analysis of antigen presentation *in vitro* and *ex vivo* (CFSE dilution assay)

For the experiments using soluble OVA or peptides, individual APCs populations were plated in 96-well plates (Nunc) at $1x10^4$ cells per well with the indicated amount of soluble EndoGrade OVA (Profos), $OVA_{257-264}$ (SIINFEKL, Ana Spec Inc.) or $OVA_{323-339}$ (kindly provided by Dr. W. Tegge, HZI) for 1h at 37°C in complete IMDM. The cells were further washed three times and resuspended in complete IMDM containing $2x10^5$ CFSE labeled OT I or OT II cells. Proliferation of T cells was analyzed by flow cytometry after 1.5 (OT I peptide) or 2.5 days of culture. Cells were stained with anti-CD4 or anti-CD8 Abs for 20 minutes, washed and resuspended in 200 µl of PBS containing Cy5-labeled 0.6µm latex beads to normalize cell numbers. Samples were analyzed until $2x10^4$ beads were collected. The number of divided cells (propidium $iodide^{lo}$, $CFSE^{lo}$, $CD4^+$ or $CD8^+$) was determined as described (128). For *ex vivo* experiments mice were injected intravenously with 1mg of OVA or 1mg of OVA together with 200µg poly I·C (Fluka). 24h later mice were sacrificed, APCs were sorted and incubated with OT I or OT II cells for 2.5 days. In some experiments

murine recombinant IFN-β (R&D Systems) was added to the cultures of T cells and APCs.

2.10 Determination of antigen uptake and processing

Sorted DCs were incubated with 62.5μg/ml of DQ-OVA (Molecular Probes) for 45 min at 37°C or on ice. DCs were then washed carefully and analyzed by FACS. For analysis of uptake of OVA-Cy5 soluble OVA was conjugated to PE-Cy5. The labeling procedure involved gel filtration as a final step for removal of low molecular mass molecules such as unbound fluorochrome. The concentration of OVA-Cy5 was determined by measurement of OD_{280}. Sorted DCs were incubated with indicated concentrations of OVA-Cy5 for 1h, than washed carefully. Uptake of fluorescent OVA was determined by FACS.

2.11 B3Z colorimetric assay

Sorted splenic cDCs (10^4cells/well) were pulsed for 1 h with various concentrations of SIINFEKL peptide, washed twice and resuspended in phenol-red free RPMI (Gibco) containing 100U/ml penicillin and 100μg/ml streptomycin, 1% FCS and 2mM L-Glutamine. DCs were then co-cultured in a 96-well U bottomed plate with $5x10^4$ B3Z cells/well overnight at 37°C (B3Z cells expresses β-galactosidase of *E.Coli* under the promoter of IL-2). Next day 150μl of supernatant was taken from each well and replaced with 150μl of PBS containing 5mM ONPG (Sigma) and 0.5% IGEPAL-20 (Sigma). The plate was than incubated at 37°C for 2 h and optical density was measured at 450 nm with wavelength correction set at 650 nm.

2.12 *In vitro* NKT cell proliferation assay

For the testing of NKT cells proliferation and activation, APC (cDCs and MZ B cells) were loaded with 1μg/ml or graded concentrations of α-galactosylceramide (αGalCer). αGalCer (Alexis Biochemicals) was not removed during the assays. Sensitized APCs ($5x10^4$) were incubated with $5x10^4$ CFSE labeled NKT cells for 16h or 2.5 days on

96-well U bottomed plates. For the last 4 hours of incubation, Brefeldin A was added to block protein secretion. First, cultured supernatants were collected to test concentration of secreted cytokines. Further, cells were stained intracellularly for IL-4 and IFN-γ. The number of divided cells (propidium iodide^{lo}, CFSE^{lo}, NK1.1^{+}) was also determined. In some experiments anti-IL-12 (10μg/ml), anti-GITR (10μg/ml), anti-ICOSL (10μg/ml) Abs and IgG isotype control (10μg/ml) were added into the APCs/NKT cells co-cultures. In one approaches mice were injected i.v. with an anti-CD40 Ab (150μg/mouse) for 4 hours.

2.13 *In vivo* NKT cell activation assay

For *in vivo* testing of NKT cell activation and cytokine production by MZ B cells and cDCs, APCs were sorted as described above, loaded *ex vivo* with 2μg/ml of αGalCer for 45 min and washed extensively. Further 5×10^5 of antigen loaded MZ B cells or cDCs were injected i.v. into CD1d$^{-/-}$ recipient mice together with purified NKT cells (1×10^7). As a control untreated MZ B cells and cDCs were injected with together with NKT cells. After 16h spleens of recipient mice were removed, splenocytes were isolated and incubated for 4h with Brefeldin A (25μg/ml) (Sigma). Further cells were stained intracellularly for IL-4 and IFN-γ.

2.14 Intracellular staining of cytokines

After 16h or 2.5 days co-culture of APCs and NKT cells, cells were incubated for the last 4 h with 25μg/ml of Brefeldin A (Sigma) or for 2h with PMA/Ionomycin and than 2h with Brefeldin A for *ex vivo* experiments. Further, cells were stained for NK1.1, CD11c (when cDCs were used as APCs) or B220 (when MZ B cells were used as APCs), fixed and permeablized according to manufacturer protocol (Cytofix/Cytoperm kit, BD) next stained for intracellular IFN-γ Pacific Blue, IL-4 PE.

2.15 Intracellular staining of Hsp70

Splenic cDCs were sorted and stained intracellularly using Cytofix/Cytoperm kit (BD Pharmingen) according to manufacturer's protocol with anti-Hsp70 (C92F3A-5) PE conjugated Ab (Stressgen).

2.16 Presentation of cell associated OVA by RAG$^{-/-}$ cDCs

Splenic cDCs from WT and RAG$^{-/-}$ mice were as described above. Further, they were plated on 96-well plates (1×10^4/well) together with UV-irradiated EG7-OVA or EL-4 cells (1×10^4) and after 1h OT I CFSE labeled cells were added. Proliferation of T cells was assessed after 2 days, according to the protocol described for soluble OVA.

2.17 Inhibition of Hsp70 by 15-deoxyspergualin (DSG)

DSG was a generous gift of Nippon Kayaku Co. Ltd. (Tokyo, Japan). Animals were injected intraperitoneally daily with 10mg/kg of DSG or PBS for 6 days before splenic cDCs were sorted and tested for their antigen presentation capacity with OT I or OT II cells in CFSE dilution assays.

2.18 Quantitative real-time PCR

Total RNA was extracted from sorted APCs using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. DNA contamination in the total RNA preparation was eliminated using DNase I (Qiagen). Oligo(dT)$_{18}$ primers and RevertAid^{TM} First Strand cDNA Synthesis kit (Fermentas) was used for reverse transcription of purified RNA. All gene transcripts were quantified by quantitative PCR with Power SYBR Green qPCR Master Mix (Applied Biosystems) and the Light Cycler apparatus (ABI PRISM Cycler Applied Biosystems).

Primers were used as follows:

RPS9 primers:

Sense, 5'-CTGGACGAGGGCAAGATGAAGC-3'

Anti-sense, 5'- TGACGTTGGCGGATGAGCACA-3'

Hsp70.1 primers (129):

Sense, 5'-AGGTGCTGGACAAGTGCCAG-3'

Anti-sense, 5'-AACTCCTCCTTGTCGGCCA-3'

C1qa primers:

Sense, 5'- ATGGACAGTGGCTGAAGATG-3'

Anti-sense, 5'- AAAACCTCGGATACCAGTCC-3'

C1qb primers:

Sense, 5'- GGTTCCTGGCTCTGATGG-3'

Anti-sense, 5'- GGTCCCCTTTCTCTCCAAAC-3'

2.19 Microarray studies

RNA isolation, cDNA preparation and DNA microarray analysis of gene expression was performed at the gene array facility of the HZI. Fluorescent images of hybridized microarrays (Affymetrix, MOE-430 version 2.0) were obtained using an Affymetrix Genechip Scanner. Microarray data were analyzed using BioConductor Suite 2.1 software. All samples were repeated two times with individually sorted cells and averaged. Data discussed here have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE12392.

Data concerning $RAG^{-/-}$ cDCs are accessible through following link:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=tpulxgoekcgysxy&acc=GSE17989

2.20 Western blot for degradation of OVA

Splenic cDCs (at least 5×10^5 per sample) were sorted from WT and $RAG^{-/-}$ mice. Cells were loaded with OVA-bio for 1h, washed extensively, lysed in RIPA buffer (100mM Tris HCl, 150mM NaCl, 1% IGEPAL-20, 0.3mM PMSF) buffer in the presence of proteases inhibitor coctail, centrifuged and boiled for 5 min in the presence of 2-mercaptoethanol. Further samples were subjected to 10% SDS-PAGE followed by transfer to polyvinylidene difluoride (PVDF) membranes (Millipore). Blocking of membranes was performed in SuperBlock (Pierce) to avoid endogenous biotin contamination present in milk. Whole OVA as well as degradation products were detected by streptavidin conjugated with horsreadish-peroxidase (BD Pharmingen). As a control, membranes were incubated with rabbit anti-actin (Sigma) primary Ab, followed by incubation with secondary anti-rabbit Ab conjugated with horsreadish-peroxidase (Sigma). All Abs were diluted in SuperBlock. The blots were developed with an enhanced chemiluminescence method (advanced ECL kit, Amersham Biosciences). Chemiluminescence was detected using the ChemiDoc XRS system (Bio-Rad).

2.21 *In vivo* treatment with antibodies

To examine the influence of soluble antibodies like IgM and IgG on the function of RAG deficient splenic cDCs, WT and $RAG^{-/-}$ mice were i.v. injected with 75µg/ml of IgM (BD Pharmingen), 75µg/ml IgG (Dianova), IgM+IgG (75µg/ml+75µg/ml) or PBS on day 0 and day7. After 21 days mice were sacrificed and splenic cDCs were sorted. The cells were loaded with soluble OVA and used as APCs in OT I assays. Proliferation of T cells was determined as mentioned before.

2.22 ELISA

ELISA was used for the detection and quantification of cytokines secreted by activated NKT cells in culture supernatants. Anti IFN-γ and IL-4 ELISA kits were purchased from Becton Dickinson and performed according to manufacturer protocol.

2.23 Annexin V staining

Staining was performed according to manufacturers protocol (BD Pharmingen, FITC Annexin V apoptosis kit). Briefly, 1×10^5 splenocytes isolated from $RAG^{-/-}$ or WT mice were resuspended in 1×Binding Buffer (BD), incubated for 15 min at RT with Annexin V (BD) in the presence of PI. Further samples were diluted with 1×Binding Buffer and analyzed by flow cytometry

2.24 Reconstitution of RAG deficient mice with B cells

WT and RAG deficient mice were injected i.v. with 5×10^6/mouse freshly sorted WT $CD19^+$ splenic B cells. 21 days after reconstitution splenic cDCs were sorted from recipient mice and used as APCs in OT I antigen presentation assay. Proliferation of T cells was determined as mentioned before.

CHAPTER III

RESULTS PART I

ABSENCE OF IFN-β IMPAIRS ANTIGEN PRESENTATION CAPACITY OF SPLENIC DENDRITIC CELLS *VIA* DOWN-REGULATION OF HSP70

[published in The Journal of Immunology 2009, July 15; 183(2): 1099-109]

Type I interferons (IFNs) play a key role in linking the innate and adaptive arms of the immune system. Although produced rapidly in response to pathogens, IFNs are also produced at low levels in the absence of infection. In the present study I demonstrate that constitutively produced IFNs are necessary *in vivo* to maintain dendritic cells (DCs) in an "antigen presentation competent" state. Conventional dendritic cells (cDCs) isolated from spleens of IFN-β or IFNs receptor (IFNAR) deficient mice exhibit a highly impaired ability to present antigen and activate naive T cells. In the absence of both IFN-β and IFNAR receptor I detected a lower number of specific MHC/peptide complexes at the surface of splenic cDCs. Microarray analysis of mRNA isolated from IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cDCs revealed diminished expression of two genes that encoded members of the heat shock protein 70 (Hsp70) family, which are required for efficient generation of stable MHC/peptide complexes expressed on the cell surface of cDCs. Consistent with this observation, pharmacological inhibition of Hsp70 in cDCs from wild type (WT) mice impaired their T cell stimulatory capacity. Similarly, the antigen presentation ability of splenic cDCs isolated from Hsp70.1/3$^{-/-}$ mice was also severely impaired in comparison to WT cDCs. Thus, constitutive IFN-β expression regulates Hsp70 levels in order to help maintain DCs in a competent state for efficient priming of effector T cells *in vivo*.

3.1 Splenic dendritic cells from IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice are impaired in T cell stimulation

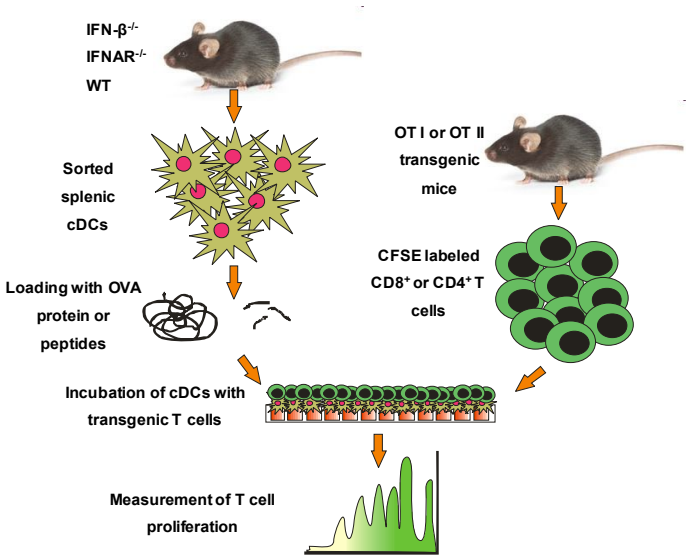
IFNs are known to be constitutively produced at low levels under non-inflammatory conditions (16). To study the influence of IFNs on antigen presentation under physiological conditions I decided to focus on freshly isolated splenic cDCs. These cells are representative of typical non migratory DCs found *in vivo* at steady state (47, 51, 130). Analyzing IFN-β and IFNAR deficient mice, I detected no differences with regard to percentage of various splenic cDCs subpopulations in mice with and without either IFN-β or IFNAR (Fig. 3.1A). Furthermore, I determined the overall number of

leukocytes in several lymph nodes and spleen. Consistently, there was no significant difference observed in comparison to WT mice (Fig. 3.1B).

Thus, I analyzed the ability of cDCs exhibiting the markers $CD11c^{hi}$, $CD11b^{+/-}$, $CD8\alpha^{+/-}$, $B220^{-}$ from spleens of WT, IFN-$\beta^{-/-}$ and $IFNAR^{-/-}$ mice to present ovalbumin (OVA) protein to CFSE labeled OT I or OT II T cells according to the protocol displayed in Scheme 3.1. When compared to WT, cDCs from IFN-$\beta^{-/-}$ and $IFNAR^{-/-}$ mice were severely impaired in their ability to activate such $CD8^{+}$ and $CD4^{+}$ T cells (Fig. 3.2A and 3.2C).

To test whether the anomaly associated with IFN-β or IFNAR deficiency affects also the presentation of preprocessed antigen, i.e. peptides, cDCs from WT, IFN-$\beta^{-/-}$ and $IFNAR^{-/-}$ mice were loaded with MHC class I or MHC class II specific peptides ($OVA_{257-264}$ (SIINFEKL) and $OVA_{323-339}$, respectively) and then incubated with CFSE labeled OT I and OT II T cells. As shown in Fig. 3.2B and 2D, T cell stimulation was also highly impaired when peptide loaded cDCs from IFN-$\beta^{-/-}$ and $IFNAR^{-/-}$ mice were used.

Throughout most of these experiments, I used bulk sorted splenic cDCs because during *in vitro* co-cultures with T cells the two distinct populations - $CD8\alpha^{+}$ DCs and $CD8\alpha^{-}$ DCs (myeloid DCs) from IFN-$\beta^{-/-}$ and $IFNAR^{-/-}$ mice were similarly impaired in their T cell stimulatory capacity compared to WT DCs (Fig. 3.3).



Scheme 3.1 *In vitro* antigen presentation assay to test the T cell stimulatory capacity of cDCs from mice defective in the type I IFN system

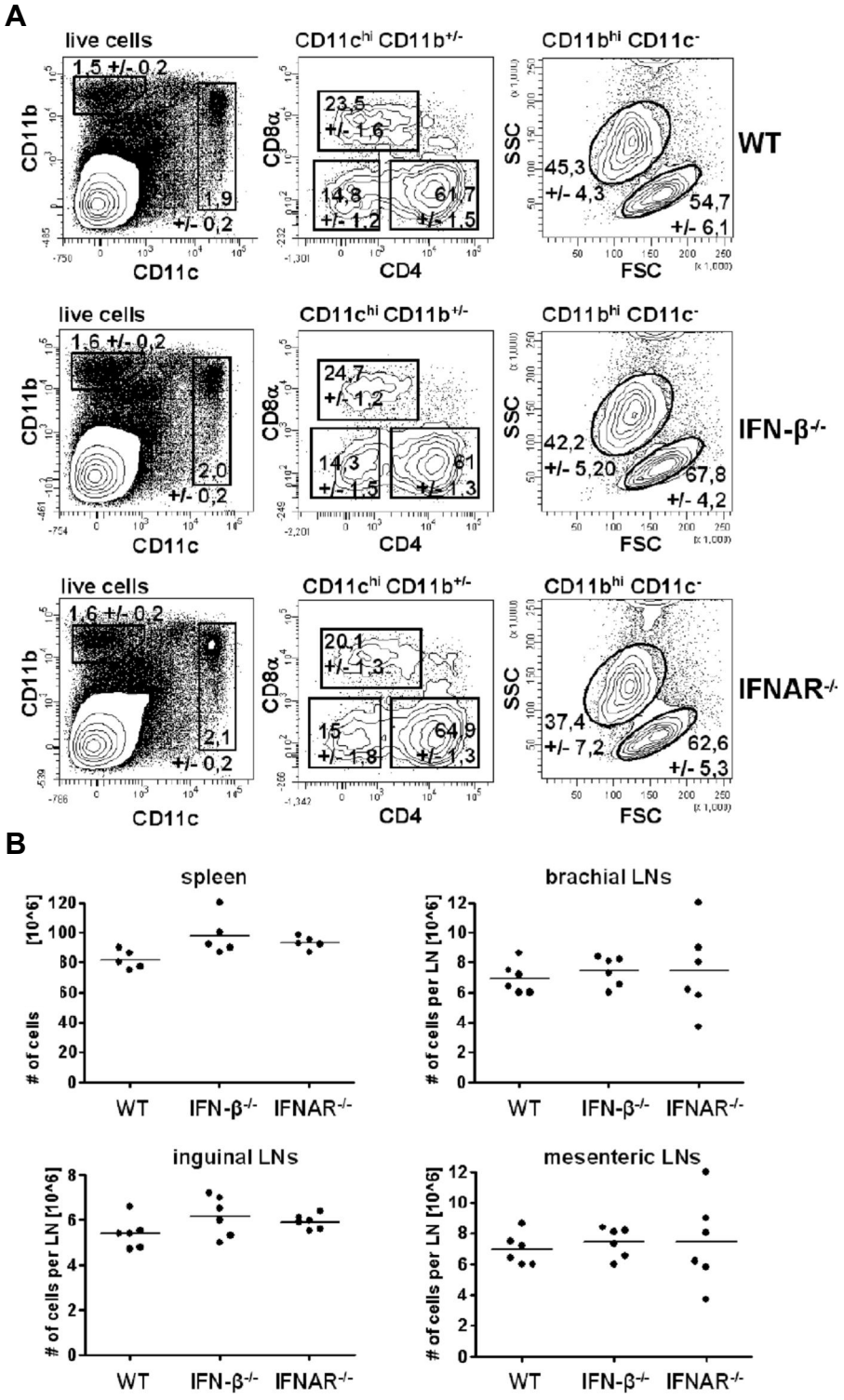


Figure 3.1 Similar percentage and frequency of different splenic cDCs populations, macrophages and granulocytes in spleens of WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice. (**A**) Splenocytes from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice were isolated and stained for following markers: CD11c, CD11b, CD4, CD8α and B220 and analyzed by flow cytometry. (**B**) Similar number of leukocytes per lymph node or spleen in WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice. Data are representative of three independent experiments with at least five mice per group.

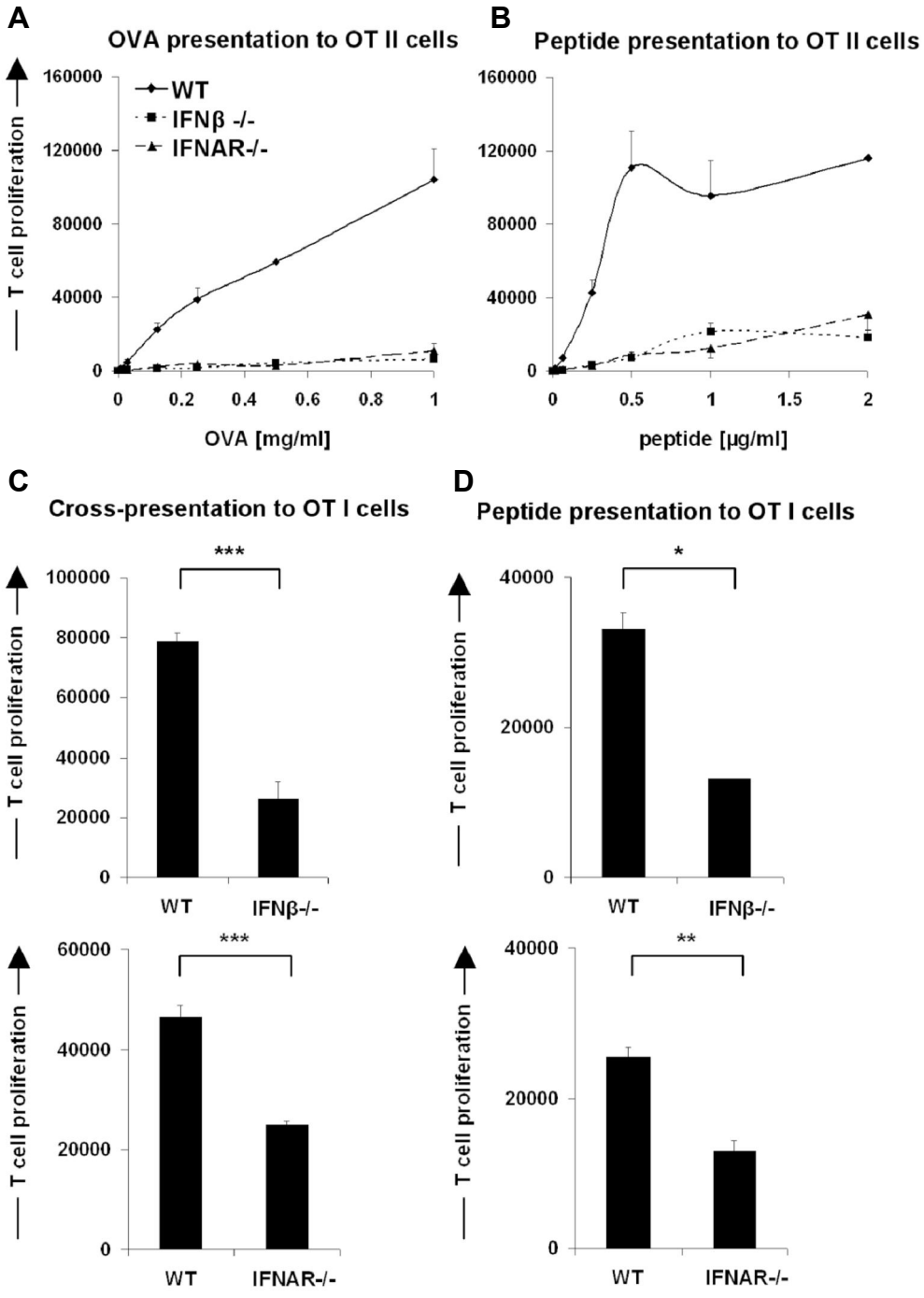


Figure 3.2 Splenic conventional dendritic cells (cDCs) from IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice are impaired in their antigen presentation capacity of soluble OVA as well as OVA derived peptides in the context of both MHC I and MHC II. The purified OT I or OT II transgenic T cells were labeled with CFSE and incubated for 1.5 days (OT I peptide) or 2.5 days with splenic cDCs (CD11c^{hi}, CD11b$^{+/-}$, CD8α$^{+/-}$, B220^{-}) in ratio 10:1. cDCs from C57BL/6 WT, IFN-β$^{-/-}$ and from IFNAR$^{-/-}$ mice were preloaded with OVA$_{257-264}$ (class I-restricted), OVA$_{323-339}$ (class II-restricted) peptides or whole OVA protein for 1h and further were washed intensively. (**A** and **B**) Antigen presentation in the context of MHC II, (**C** and **D**) Antigen presentation in the context of MHC I. OVA concentration 250µg/ml, OVA$_{257-264}$ peptide concentration 10ng/ml. The proliferative response of T cells was enumerated by flow cytometry. Data are representative of at least three mice for WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ in multiple independent experiments. Statistical significance was determined using the paired Student's t test. * P<0.05; ** P<0.01; *** P<0.005

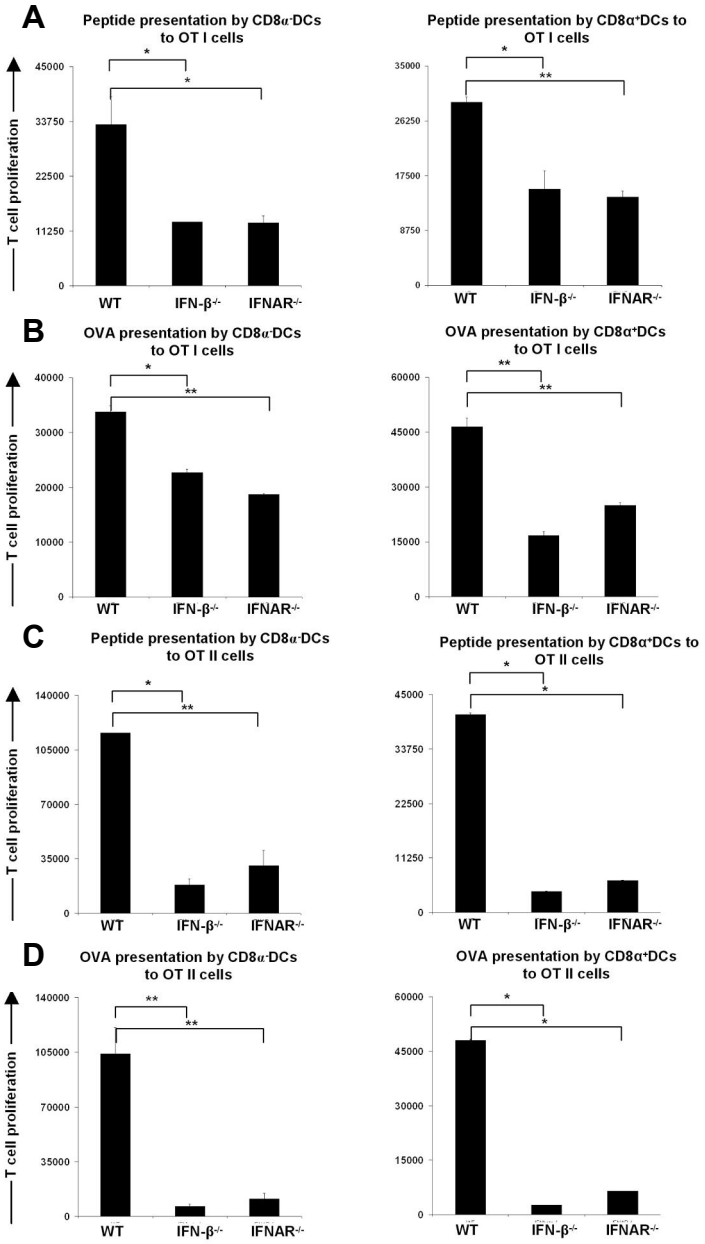


Figure 3.3 Two distinct populations of splenic conventional DCs (cDCs) – CD8α$^+$DCs and CD8α$^-$ DCs from IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice are similarly impaired in their ability to activate naive T cells in comparison to WT DCs. The purified OT I or OT II transgenic T cells were labeled with CFSE and incubated for 1.5 days (OT I peptide) or 2.5 days with splenic CD8α$^+$DCs (CD11c^{hi}, CD11b$^+$, CD8α$^+$, B220$^-$) or CD8α$^-$DCs (CD11c^{hi}, CD11b$^-$, CD8α$^+$, B220$^-$) in ratio 10:1. Both DCs populations from C57BL/6 WT, IFN-β$^{-/-}$ and from IFNAR$^{-/-}$ mice were preloaded with OVA$_{257-264}$ (class I-restricted), OVA$_{323-339}$ (class II-restricted) peptides or whole OVA protein for 1h and further were washed intensively. (**A** and **B**) Antigen presentation in the context of MHC I, (**C** and **D**) Antigen presentation in the context of MHC II. OVA concentration 250μg/ml (MHC I), OVA concentration 500μg/ml (MHC II), OVA$_{257-264}$ peptide concentration 10ng/ml, OVA$_{323-339}$ peptide concentration 2μg/ml. The proliferative response of T cells was enumerated by flow cytometry. Data are representative of at least three mice for WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ in three independent experiments. Statistical significance was determined using the paired Student's t test. * P<0.05; ** P<0.01; *** P<0.005

3.2 Deficiency in IFNs does not impair survival of cDCs *in vitro*

As IFNs provide cellular survival signals under certain conditions (26, 131), I first wanted to test whether the reduced ability to stimulate T cells might be due to lower survival of cDCs from IFN-$\beta^{-/-}$or IFNAR$^{-/-}$ mice during the *in vitro* T cell stimulation assay. Splenic cDCs sensitized with OVA protein were incubated with OT I or OT II cells. After 16 and 32 hours the percentage of live cDCs was assessed by propidium iodide (PI) exclusion. WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cDCs were equally viable under these conditions (Fig. 3.4A, 3.4B). Thus, the reduced ability to stimulate T cells *in vitro* was not due to lower survival of IFN-$\beta^{-/-}$ cDCs.

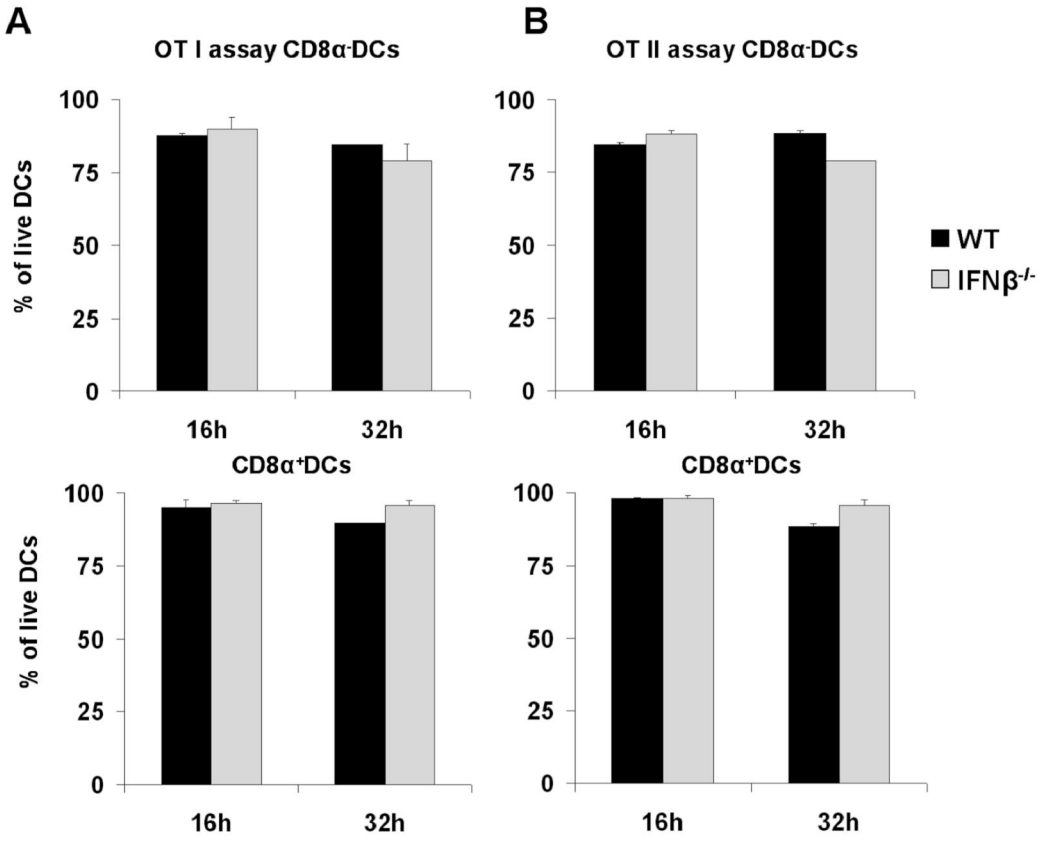


Figure 3.4 Impaired antigen presentation capacity of splenic cDCs from IFN-$\beta^{-/-}$ mice is not due to their impaired viability. Survival of splenic cDCs from WT and IFN-$\beta^{-/-}$ mice during incubation with (**A**) OT I or (**B**) OT II cells. Splenic cDCs from WT and IFN-$\beta^{-/-}$ mice were loaded with OVA protein (250µg/ml OVA for OT I and 500µg/ml OVA for OT II), washed and incubated with transgenic T cells for 16h and 32h. In indicated time points cells were collected, stained for CD11c, CD11b, CD8α and B220. Percentage of live CD8α^{+}DC or CD8α^{-}DCs was appointed by PI exclusion. Data are representative of at least three mice for WT and IFN-$\beta^{-/-}$ in two independent experiments.

3.3 Impaired stimulatory capacity of cDCs can be restored by supplementation with recombinant IFN-β *in vitro* or induction of IFNs with poly I·C *in vivo*

Next, I asked whether exogenous administration of IFNs could restore the impaired T cell stimulatory capacity of IFN-$\beta^{-/-}$ cDCs *in vitro*. Titration of murine recombinant IFN-β (rIFN-β) into co-cultures of IFN-$\beta^{-/-}$ cDCs and T cells showed that low amounts (0.1U/ml) could completely restore the impaired T cell stimulatory function (Fig. 3.5A). However, probably due to activation of negative feedback mechanisms, addition of higher concentrations of rIFN-β (5-500U/ml) to the co-cultures failed to restore T cell stimulatory ability of cDCs (Fig. 3.5A and data not shown). These results support the argument that the low levels of IFN-β produced at steady state are well optimized for maintaining of cDCs in antigen presentation competent state.

Nevertheless, in such a situation it is difficult to exclude that exogenous rIFN-β influenced T cell proliferation. A direct effect of IFNs on T cells has been well documented (24, 26) although it only partially could explain my results. I could show that IFNAR$^{-/-}$ cDCs which are able to produce IFN-β (data not shown), are still inefficient in activating a T cell response (Fig. 3.2). In addition, when such co-cultures are complemented with recombinant IFN-β the inefficiency of T cell activation remained (Fig. 3.5B). This clearly demonstrates that steady state production of IFN-β is required for maintenance of proper cDC function.

Furthermore, I tested whether triggering IFNs-α *in vivo* could compensate the impaired development of T cell stimulatory capacity of cDCs from IFN-$\beta^{-/-}$ mice. WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were intravenously (i.v.) injected with OVA alone or OVA together with polyinosinic-polycytidylic acid (poly I·C) a well known stimulator of IFNs. After 24h, splenic cDCs were sorted and tested for their ability to activate the proliferation of OT I or OT II T cells. Data depicted in Fig. 3.5C show that IFNs-α induction by poly I·C compensated for the lack of IFN-β during cDC development *in vivo* and partially recovered their function. As expected, *in vivo* administration of poly I·C did not improve the stimulatory function of splenic cDCs from IFNAR$^{-/-}$ mice as they are completely unresponsive to IFNs signaling (Fig. 3.5D).

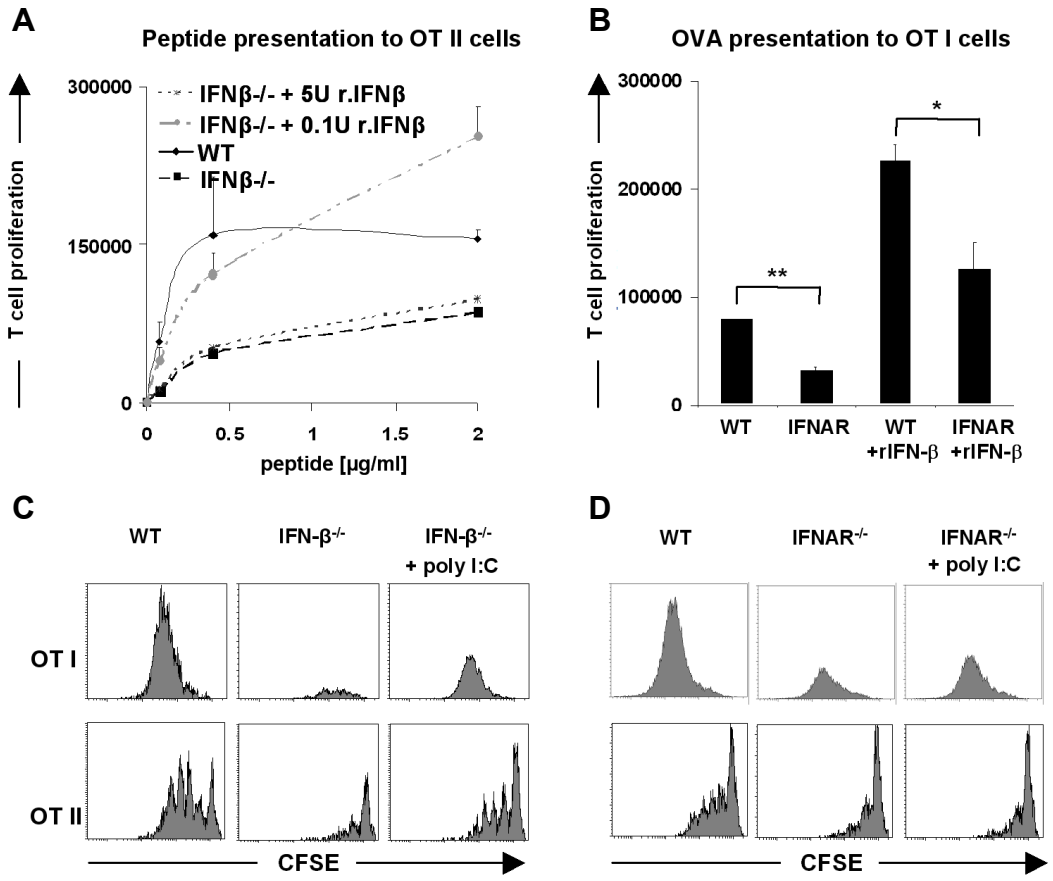


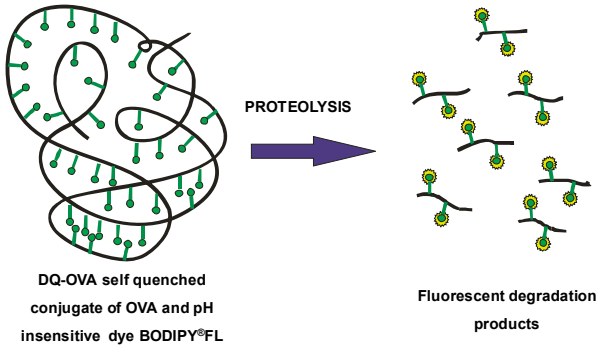
Figure 3.5 IFN-β is required *in vitro* during cDC-T cell contact as well as *in vivo* during cDCs development. (**A**) Addition of low amounts of murine recombinant IFN-β (0.1U/ml) leads to efficient recovery of impaired ability to present antigen by splenic cDCs from IFN-$\beta^{-/-}$ mice. OT I and OT II cells after purification were labeled with CFSE. Splenic cDCs from WT and IFN-$\beta^{-/-}$ mice were sorted out from spleens of at least three mice, loaded with indicated concentration of OVA peptide for 1h, washed intensively and co-cultured with T cells for 2.5 days in presence of recombinant murine IFN-β. (**B**) Enhanced proliferation of T cells accompanying impaired function of IFNAR$^{-/-}$ cDCs in the presence of exogenously added low amounts of rIFN-β. Splenic cDCs from WT and IFNAR$^{-/-}$ mice were sorted out from spleens of at least three mice per group, loaded with 250µg/ml of OVA protein for 1h, washed intensively and co-cultured with OT I T cells for 2.5 days in presence of 0.1U/ml of recombinant murine IFN-β. (**C** and **D**) *In vivo* induction of IFNs by poly I·C leads to partial restoration of function of splenic cDCs from IFN-$\beta^{-/-}$ mice. WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were treated with poly I·C together with OVA or OVA alone, 24h later splenic cDCs were sorted out and incubated *in vitro* with CFSE labeled OT I or OT II cells for 2.5 days. The proliferative response of T cells was enumerated by flow cytometry. Data are representative of two independent experiments.

I also tested the T cell stimulatory capacity of DCs differentiated *in vitro* by incubating bone marrow cells with IL-4 and GM-CSF (BMDCs). After 8 days of culture I obtained around 80% CD11c positive cells from WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice. Here, WT and IFN-$\beta^{-/-}$ or IFNAR$^{-/-}$ BMDCs were comparable in their ability to stimulate the proliferation of T cells (data not shown). This suggests that the influence

of IFNs observed *ex vivo* is greatly dependent on the overall stimulatory context under which the DCs develop.

3.4 IFN-β$^{-/-}$ and IFNAR$^{-/-}$ cDCs display normal antigen capture and processing

A differential ability to acquire and process soluble antigen could account for the diminished stimulatory capacity of cDCs in the absence of the IFNs system. Therefore, the efficiency of splenic cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice to take up and degrade soluble OVA was assessed. I used DQ-OVA, which generates fluorescent byproducts upon degradation (Scheme 3.2). As shown in Fig. 3.6, splenic cDCs from all groups acquired and generated comparable amounts of fluorescent DQ-OVA products. This was true for different DQ-OVA concentrations tested (data not shown). Thus, changes in antigen uptake and degradation could not account for the impaired T cell stimulatory capacity of cDCs from IFN-β$^{-/-}$ or IFNAR$^{-/-}$ mice.



Scheme 3.2 Mechanism of action of DQ-OVA, results presented on Fig. 3.6.

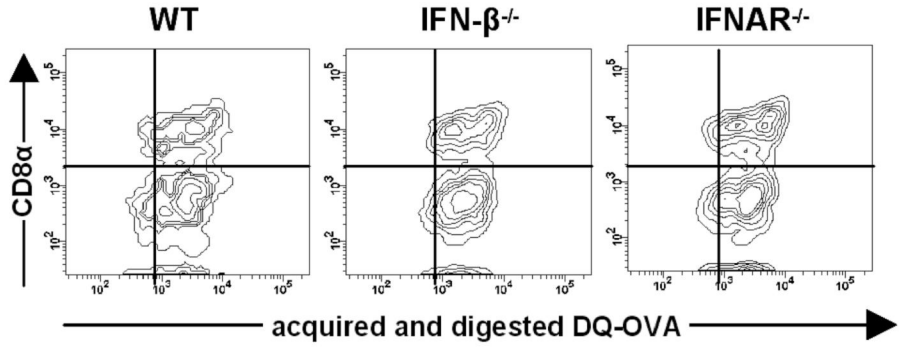


Figure 3.6 Lack of IFN-β and IFNs signaling has no influence on proper uptake and processing of soluble antigen by splenic cDCs. Splenic cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice were sorted out and incubated for 1h with 62.5µg/ml of DQ-OVA. Further cells were washed and BODIPY fluorescence was measured using flow cytometry. Density plots show DQ-OVA proteolysis by splenic cDCs. Data are representative of three independent experiments and of at least three mice per group.

3.5 Similar expression of MHC and co-stimulatory molecules on cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice

The maturation status of DCs is known to be a fundamental factor for proper T cell stimulation. One of the mechanisms by which constitutive IFNs signaling could influence T cell stimulation is to enhance expression of MHC or adhesion and co-stimulatory molecules on the surface of DCs (21). However, analysis of splenic CD8α^{+} and CD8α^{-} cDCs for MHC I and MHC II as well as co-stimulatory or adhesion molecules like CD86, CD80, CD40 and ICAM-1 indicated no significant differences between WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice in both cDC populations (Fig. 3.7). Therefore, the impaired function of splenic cDCs from IFN-β and IFNAR deficient mice was not due to lower expression of such surface molecules.

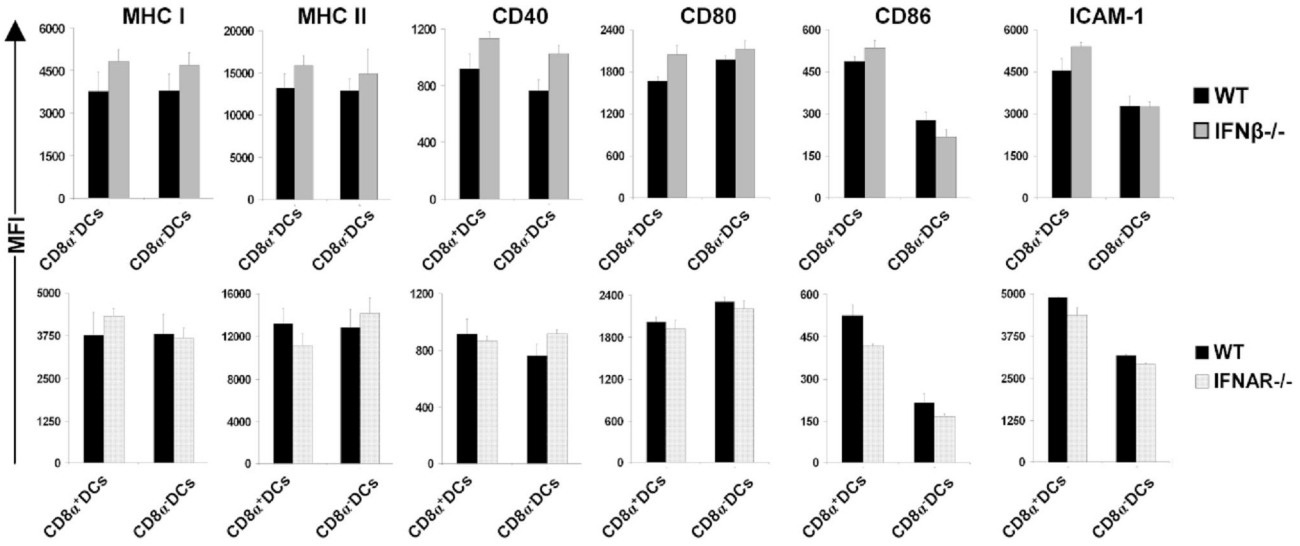


Figure 3.7 Similar surface phenotype of splenic cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice. Please see description on the next page.

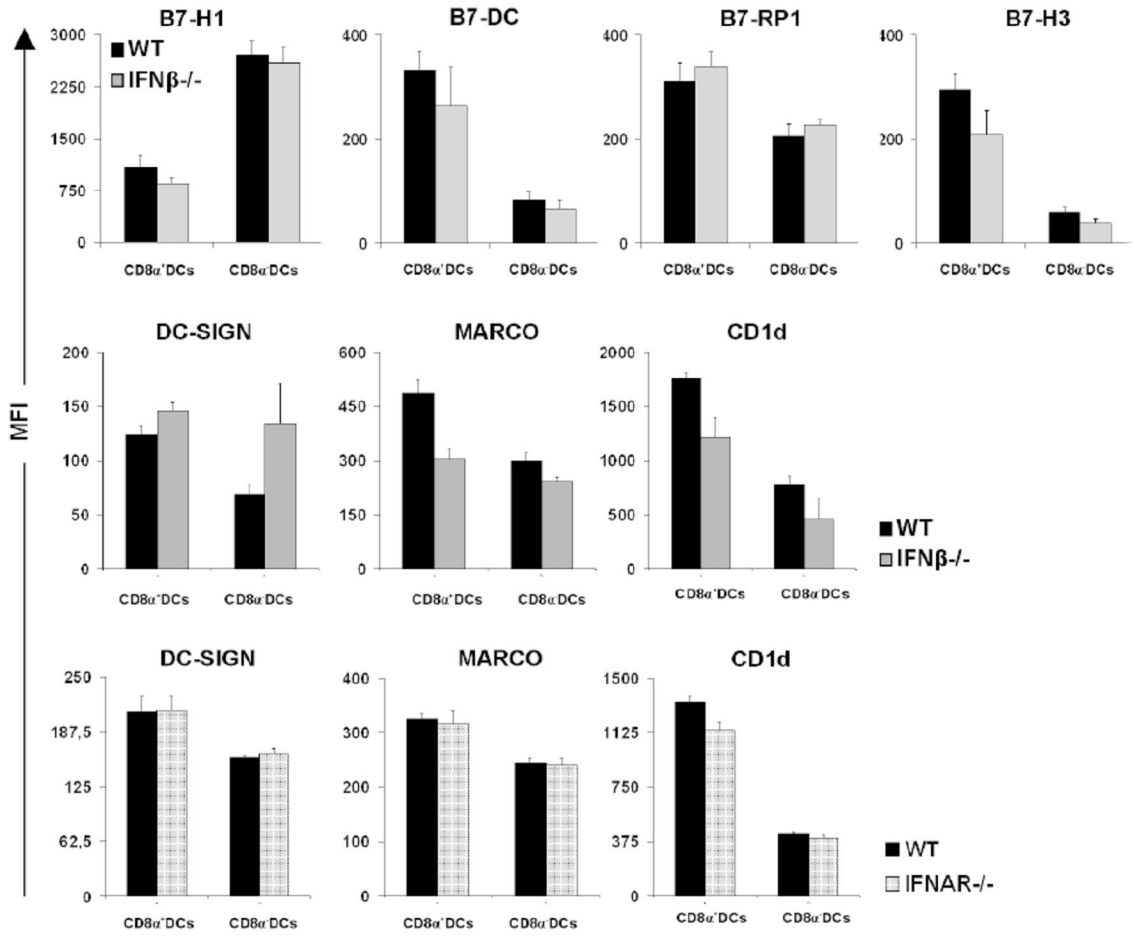
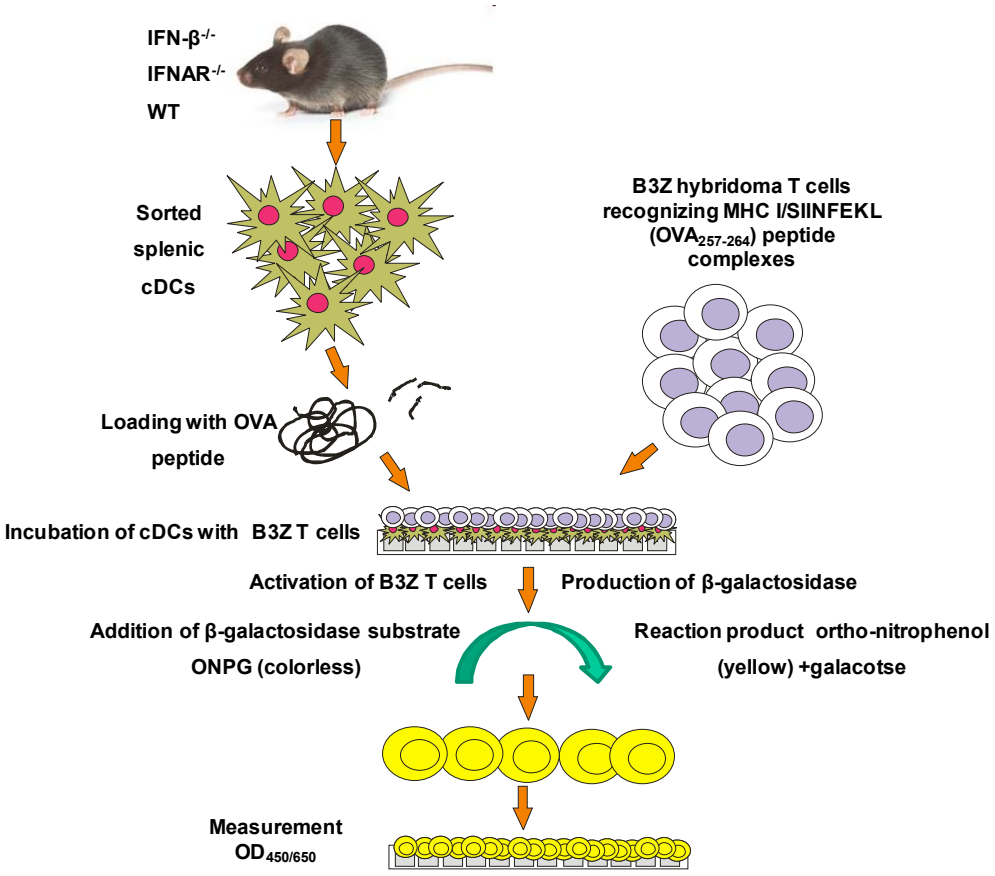


Figure 3.7 Similar surface phenotype of splenic cDCs from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice. Splenocytes isolated from C57BL/6 WT, IFN-β$^{-/-}$ or IFNAR$^{-/-}$ mice were stained and gated on the basis of CD11c, CD11b, CD8α and B220 on two populations of cDCs. Graphs show MFI (Mean Fluorescence Intensity) values for expression of indicated markers. MFI of each marker was measured for at least three mice per group. Data are representative of five independent experiments.

3.6 An intact IFNs system is required for the efficient formation of stable MHC/peptide complexes at the surface of splenic cDCs

The fact that an impaired T cell stimulatory capacity of cDCs from IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice was also found for the presentation of peptides, not requiring further processing, as well as unimpaired DQ-OVA degradation suggested that the phenotype of IFN-β$^{-/-}$ and IFNAR$^{-/-}$ cDCs was most likely due to a defect in peptide presentation rather than antigen processing steps. Therefore, I decided to study the MHC/peptide complexes on the surface of cDCs. I employed B3Z cells, a H-2·K^{b} restricted T cell hybridoma specific for the OVA epitope SIINFEKL ($OVA_{257-264}$), which upon T cell

receptor (TCR) activation expresses β-galactosidase (125). The activation of B3Z cells, being a hybridoma, is independent of co-stimulation (132). Thus, their activation should only be dependent on the concentration of MHC I/peptide complexes recognizable by the TCR. Splenic cDCs from WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were loaded with $OVA_{257-264}$ peptide and tested for their ability to activate B3Z cells (Scheme 3.3). As shown in Fig. 3.8A and 3.8B, cDCs from mice deficient in IFN-β or IFNAR exhibited lower stimulatory capacity. These results confirmed that the impaired function of splenic cDCs from such mice is not due to lower levels of co-stimulatory molecules, but strongly suggested that the defect was in the process of MHC I/peptide complex formation. By using the 25-D1.16 antibody which recognizes SIINFEKL bound to the H-2·K^{b} molecule, according to the Scheme 3.4 (126), I confirmed this interpretation. DCs from either IFN-$\beta^{-/-}$ or IFNAR$^{-/-}$ mice had lower levels of MHC I/SIINFEKL complexes compared to cDCs from WT mice (Fig. 3.8C).



Scheme 3.3 Activation of B3Z hybridoma T cells, results on Fig. 3.8A, B.

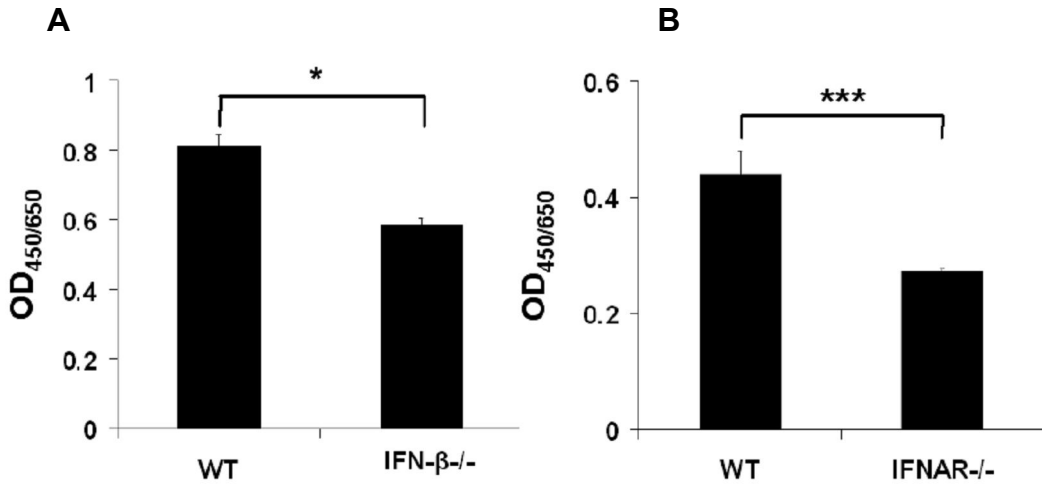
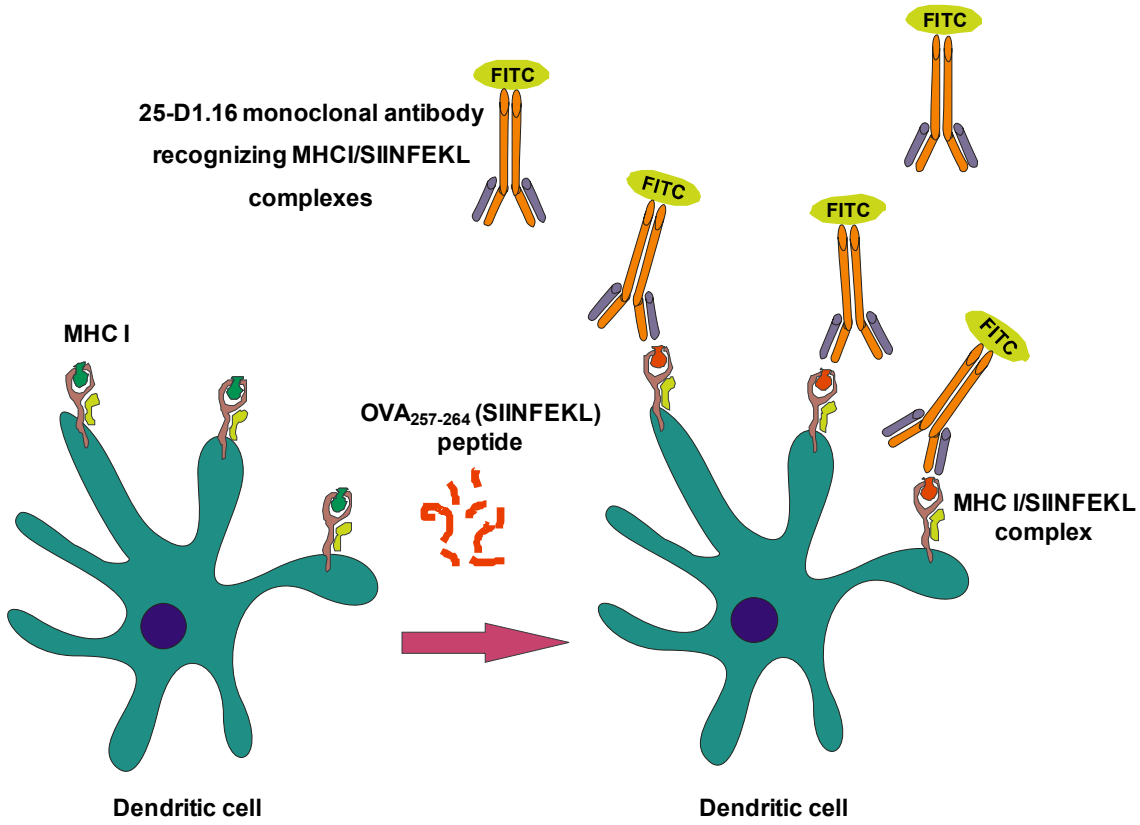


Figure 3.8 (A, B) Splenic cDCs from IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice form lower levels of MHC I/SIINFEKL complexes. Splenic cDCs were sorted out from spleens of WT, IFN-β$^{-/-}$ or IFNAR$^{-/-}$ mice, pulsed with SIINFEKL peptide ($OVA_{257-264}$) for 1h, washed intensively and (**A, B)** Co-cultured with the SIINFEKL/H-2K^{b} restricted B3Z hybridoma T cells for 24h. Cells were then lysed and monitored for LacZ expression by the introduction of ONPG substrate. Optical density was measured at 450nm with wavelength correction set at 650 nm. Results are representative of at least three mice for WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ in three independent experiments. Statistical significance was determined using the paired Student's t test. * P<0.05; *** P<0.005



Scheme 3.4 Staining with 25-D1.16 monoclonal antibody, results on Fig. 3.8C.

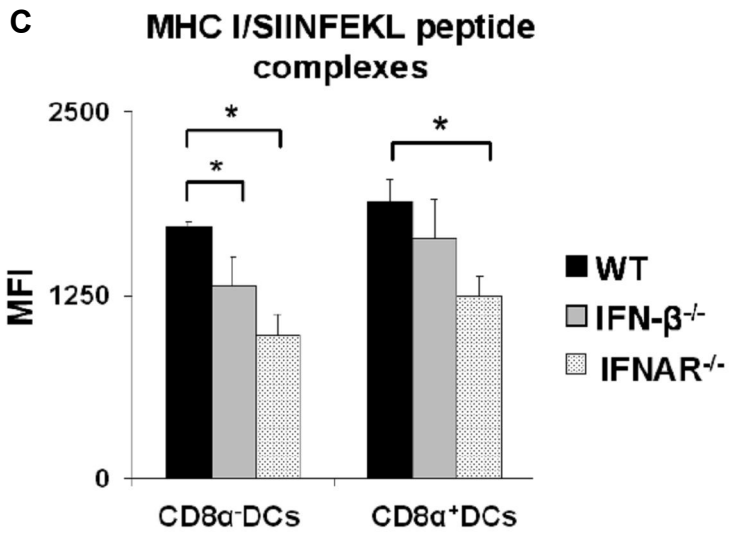


Figure 3.8 (C) Splenic cDCs from IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice form lower levels of MHC I/SIINFEKL complexes. (**C**) cDCs were stained with 25-D1.16 antibody, MFI was measured by flow cytometry, graphs show values for 100ng/ml of SIINFEKL peptide, cells were gated on CD8α^{+}DCs and CD8α^{-}DCs. Results are representative of at least three mice for WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ in three independent experiments. Statistical significance was determined using the paired Student's t test. * P<0.05; *** P<0.005

3.7 Deficiencies in the IFNs system leads to decreased expression of Hsp70 in splenic cDCs

To understand the molecular basis for the decreased formation of MHC/peptide complexes, splenic DCs RNA from WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ mice was analyzed by microarrays for expression of genes known to be involved in antigen processing and presentation, co-stimulation or IFNs response. Extensive analysis of the microarrays indicated that most of these genes were unaltered in cDCs from the knockout mice. The only dramatic difference found was in the expression of the heat shock protein Hsp70.1 and Hsp70.3 genes. The expression of these two genes was significantly lower in cDCs from IFN-β$^{-/-}$ (approximately 15-20 fold down-regulated in comparison to WT) and IFNAR$^{-/-}$ (approximately 75-150 fold down-regulated in comparison to WT) mice (Fig. 3.9A).

To verify the above findings, I first stained for intracellular Hsp70 protein. As shown in Fig. 3.10 levels of Hsp70 were indeed lower in IFN-β$^{-/-}$ and IFNAR$^{-/-}$ cDCs compared to WT cDCs. Most likely due to presence of other highly homologous members of the Hsp70 family and the low sensitivity of the antibody, differences were not very pronounced.

However, in confirmation of the microarray data, transcriptional levels of Hsp70.1 were severely decreased in DCs from IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice in comparison to WT (Fig. 3.9B). Moreover, treatment with low amounts of rIFN-β (0.1U/ml) increased Hsp70 levels in both WT and IFN-$\beta^{-/-}$ DCs, whereas treatment with 5U/ml of rIFN-β did not markedly change the Hsp70 levels (Fig. 3.9C). This correlates well with the functional restoration of IFN-$\beta^{-/-}$ cDCs at low, but not at high concentrations of rIFN-β. Consistent with this finding, 24h of poly I·C administration up-regulated Hsp70.1 levels in WT as well as in IFN-$\beta^{-/-}$ cDCs (Fig. 3.9D).

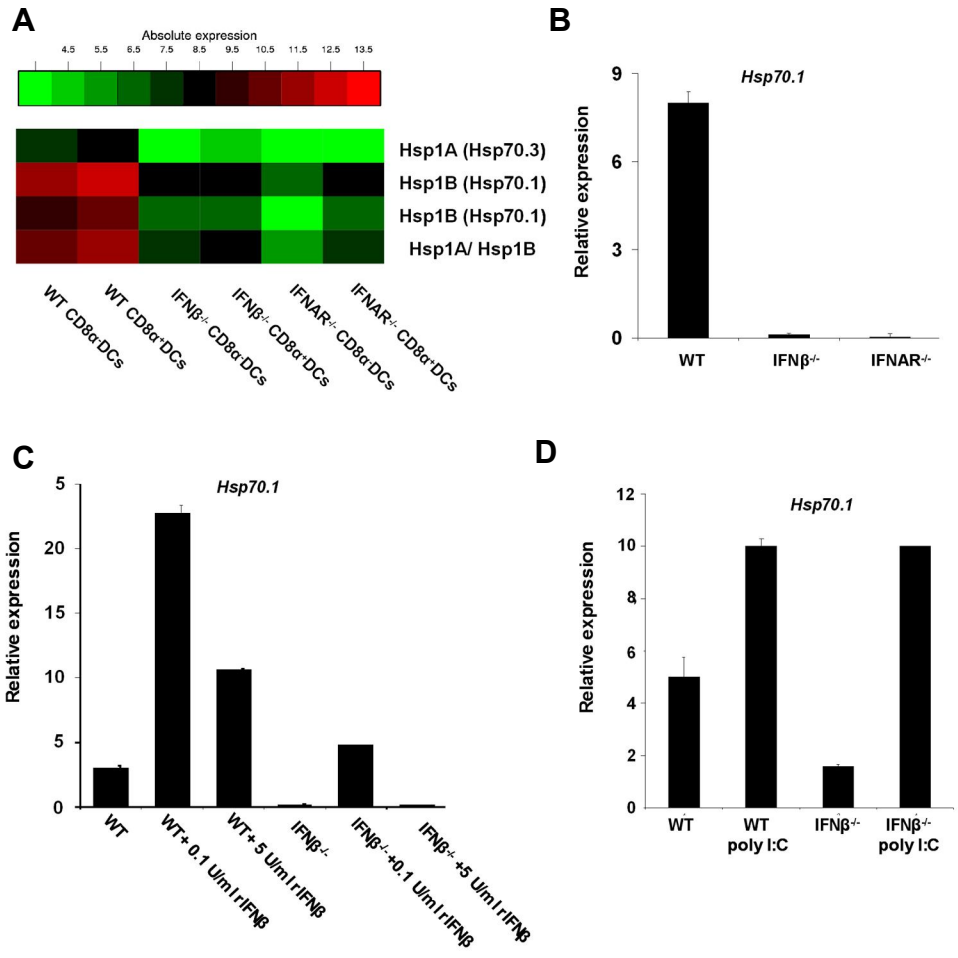


Figure 3.9 Microarray analysis of splenic cDCs from WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice showed down-regulation of heat shock protein 70.1 and 70.3 (Hsp70.1 and Hsp70.3) in knockout mice. (**A**) Affymetrix gene array analysis of splenic cDCs. RNA was prepared from sorted splenic CD8α^{+}DCs and CD8α^{-}DCs from WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice. All samples were repeated twice with individually sorted cells and averaged**.** (**B**) Quantitative real-time PCR (qRT-PCR) of RNA from splenic cDCs sorted from WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice. (**C**) qRT-PCR of RNA from splenic cDCs untreated and *in vitro* treated with 0.1U/ml and 5U/ml of murine recombinant IFN-β for 3h. (**D**) qRT-PCR of RNA from splenic cDCs 24h after administration of poly I·C. Results are representative of at least three from WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice in three independent experiments.

3.8 Inhibition of Hsp70 by 15-deoxyspergualin leads to impairment of antigen presentation

To test for a possible causative link between Hsp70 expression and antigen presentation, I used 15-deoxyspergualin (DSG), a pharmacological inhibitor of Hsp70. DSG is a synthetic derivative of spergualin from *Bacillus laterosporus* and binds to

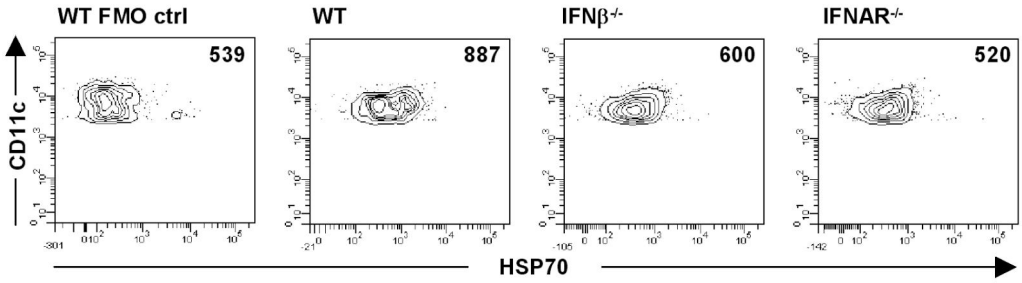


Figure 3.10 Lower amounts of Hsp70 protein in IFN-β and IFNAR deficient cDCs in comparison to WT cDCs. Intracellular staining of Hsp70 protein in sorted splenic cDCs from WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice. Results are representative of at least three mice per group in three independent experiments. FMO (fluorescence minus one) control. Values represent MFI for indicated group.

Hsp70 and Hsp90 (105, 133, 134). Therefore, I treated mice for 6 days with DSG and then tested the splenic cDCs of such mice for Hsp70 expression. Intracellular staining revealed that DSG treatment led to partial reduction of Hsp70 level in WT cDCs (Fig. 3.11A). In contrast, expression of surface molecules involved in T cell stimulation was not affected by this treatment (Fig. 3.12).

Then I tested cDCs isolated from DSG treated mice for their capacity to stimulate $CD4^+$ and $CD8^+$ T cells. Clearly, cDCs from DSG treated mice exhibited a reduced ability to stimulate OT I or OT II T cells compared to cDCs from untreated mice, independent of whether protein or peptides were used as antigen (Fig. 3.11B). This was consistent with the claims that DSG abrogates the ability to present antigen in the context of both MHC I and MHC II (105, 133-135).

In addition, compared to untreated control, cDCs from DSG treated mice revealed lower surface levels of MHC/peptide complexes (Fig. 3.11C) suggesting that Hsp70 is necessary for efficient formation of MHC/peptide complexes. Thus, by supporting the expression of Hsp70, constitutive IFN-β expression *in vivo* helps to maintain cDCs in a primed and competent state for antigen presentation.

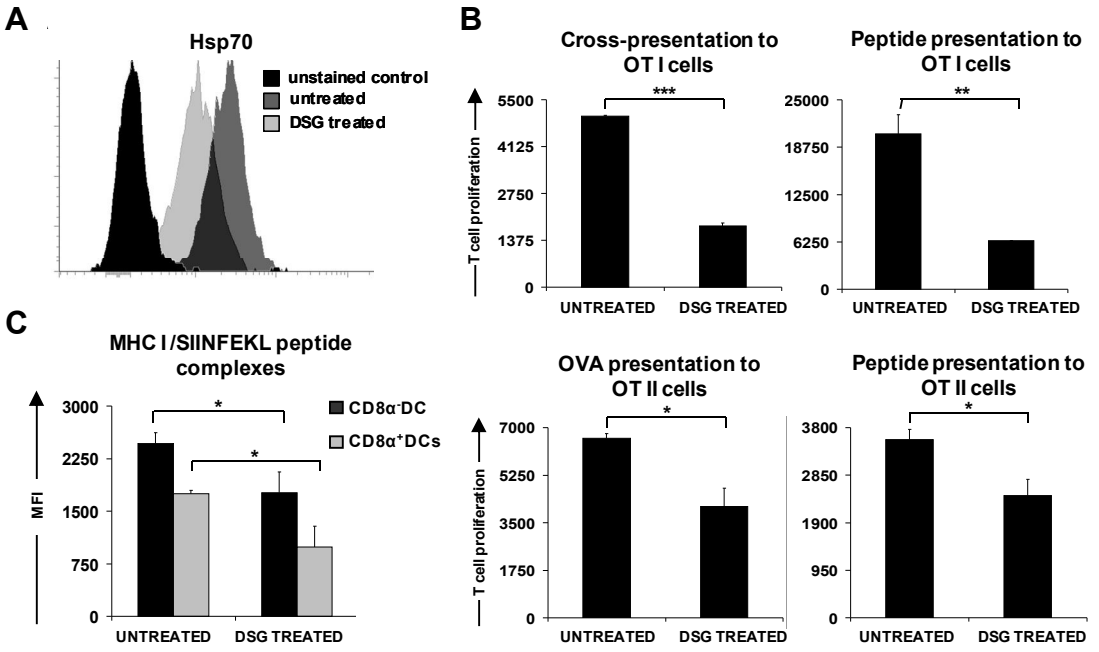


Figure 3.11 ***In vivo*** **inhibition of Hsp70 by 15-deoxyspergualin leads to impaired antigen presentation by splenic cDCs.** (**A**) Intracellular staining of Hsp70 in splenic cDC from mice untreated and treated with 15-deoxyspergualin. (**B**) Antigen presentation assay with splenic cDCs from untreated mice and mice treated with 15-deoxyspergualin. Splenic cDCs were incubated with purified, CFSE labeled OT I or OTII cells for 1.5 days (class I restricted peptide) and 2.5 days. $OVA_{257-264}$ peptide concentration 10ng/ml, $OVA_{323-339}$ 2μg/ml, OVA protein 1mg/ml (class II presentation) or 500μg/ml (class I presentation). The proliferative response of T cells was enumerated by flow cytometry. Data are representative of at least five mice for WT untreated and DSG treated in three independent experiments. (**C**) DCs from untreated and DSG treated mice were loaded for 1h with 100ng/ml of SIINFEKL peptide, washed and stained with 25-D1.16 antibody recognizing MHC I/SIINFEKL peptide complexes. Graph shows MFI values for five mice per group. Data are representative of two independent experiments. Statistical significance was determined using the paired Student's t test. * $P<0.05$; ** $P<0.01$; *** $P<0.005$

3.9 Splenic dendritic cells from Hsp70.1/3$^{-/-}$ mice are impaired in T cell stimulation

In order to explicitly demonstrate the involvement of the Hsp70.1 and Hsp70.3 proteins in antigen presentation and thus confirm that in the absence of IFN-β or IFNs signaling down-regulation of Hsp70 result in impaired antigen presentation I utilized Hsp70.1/3 double knockout mice (122). The surface phenotype of cDCs from Hsp70.1/3 $^{-/-}$ mice appeared to be very similar to the surface phenotype of cDCs from WT (data not shown).

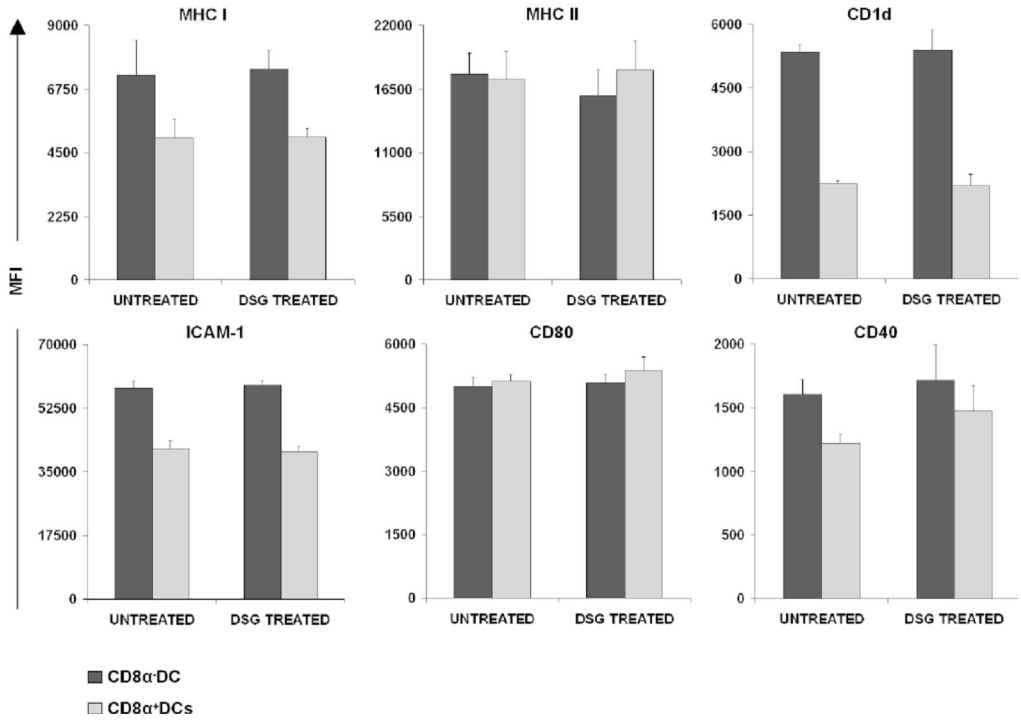


Figure 3.12 Unaltered surface phenotype of splenic cDCs after DSG treatment. Splenocytes isolated from C57BL/6 WT mice were untreated or treated for 6 days with 10mg/kg of DSG and stained for indicated surface markers. Graphs show MFI values of expression of appropriate marker. MFI was measured for at least five mice per group. Data are representative of three independent experiments.

To test the antigen presentation capacity of Hsp70.1/3 $^{-/-}$ cDCs, cells were sorted and loaded with appropriate OVA peptides or whole protein and incubated with OT I or OT II transgenic T cells. The results clearly show that cDCs from Hsp70.1/3$^{-/-}$ are impaired in their ability to present OVA derived peptides as well as whole protein to naive T cells when compared to WT cDCs (Fig. 3.13*)*. This further substantiates my finding that down-regulation of Hsp70 in the absence of IFN-β or IFNs can alter antigen presentation. The discovery that IFNs signaling regulates MHC/peptide complex formation by Hsp70 proteins highlights a hitherto unrecognized mechanism *via* which IFNs might regulate presentation of self antigens in the steady state and has, therefore, important consequences for our understanding of how regular homeostatic conditions are maintained in the immune system.

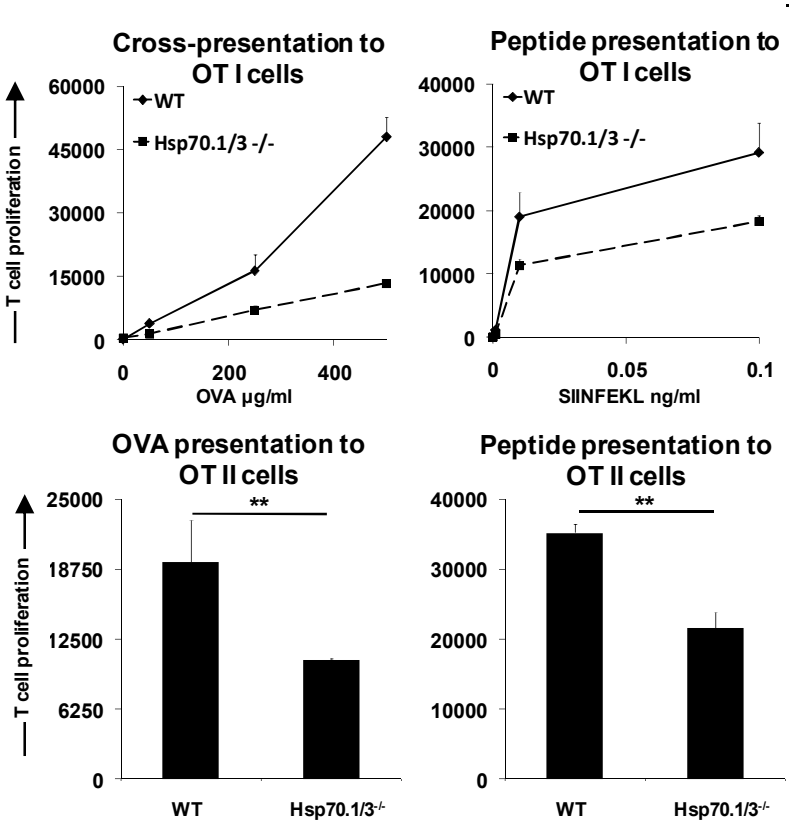


Figure 3.13 Knockout of Hsp70.1 and Hsp70.3 leads to impaired antigen presentation by splenic cDCs. Antigen presentation assay with splenic cDCs sorted from Hsp70.1/3$^{-/-}$ and WT mice. Cells were loaded with appropriate OVA derived peptides or whole OVA, washed and incubated *in vitro* with CFSE labeled OT I or OTII cells for 1.5 days (class I restricted peptide) and 2.5 days. $OVA_{323-339}$ peptide concentration 2µg/ml, OVA protein 1mg/ml (bottom panel, MHC II presentation). The proliferative response of T cells was enumerated by flow cytometry. Data are representative of three mice for Hsp70.1/3$^{-/-}$ and WT in two independent experiments. Statistical significance was determined using the paired Student's t test. * $P<0.05$; ** $P<0.01$; *** $P<0.005$

CHAPTER IV

RESULTS PART II

INFLUENCE OF B CELLS AND IMMUNOGLOBULINS ON THE ANTIGEN PRESENTATION CAPACITY OF SPLENIC DENDRITIC CELLS

The function of T and B cells is strongly influenced by DCs. For instance, it is well established that DCs and T cells have to interact to initiate adaptive immune responses. In addition, B cells need T cell help to start their major function to produce antibodies, but direct presentation of antigen to B cells has also been demonstrated (136). On the other hand activated T cells provide feedback signals to DCs and induce their maturation. Under these circumstances, DCs lose their adhesive and phagocytic capacities *via* mechanism called "T cell-mediated terminal maturation" (137). This maturation is initiated by cytokines produced by T cells as well as by direct cell-cell interactions, that include CD40/CD40L pathway.

B cells also exhibit profound regulatory effects on the function of DCs. For example, in the absence of B cells DCs have an impaired capacity to induce differentiation of IL-4 producing T cells (138, 139).

The goal of this part of the thesis was to characterize DCs from mice that lack T and B cells, like RAG (recombination activated gene) deficient mice, and to evaluate the influence of mature B and T cells on the antigen presenting ability of splenic cDCs. The results demonstrated, that cDCs from $RAG^{-/-}$ mice were impaired in their ability to present soluble antigen. I could show that this was due to their highly aggressive protein degradation compartments. In contrast, cDCs from $RAG^{-/-}$ mice were very efficient in presenting cell associated antigens, like antigen associated with apoptotic cells. This might be explained by the fact, that $RAG^{-/-}$ cDCs express high levels of C1q complement members, which are responsible for the clearance of apoptotic cells. Finally, I showed that cDCs from $RAG^{-/-}$ mice express increased levels of Fcγ receptors (FcγR). Consequently, administration of B cells or soluble immunoglobulins into $RAG^{-/-}$ mice recovered the impaired presentation capacity of splenic cDCs for soluble antigen. Taken together, I could show here that the cellular composition of the spleen and antibodies in the blood dramatically influence the character and function of cDCs.

4.1 Splenic cDCs from RAG deficient mice exhibit similar surface phenotype to WT cDCs

Products of the RAG-1 and RAG-2 genes are responsible for initiation of a complex series of DNA rearrangements collectively called variable-diversity-joining (V(D)J) recombination. This process is responsible for the diverse repertoire of antigen receptors present on B and T cells (140). Deletion of such genes leads to an arrest of B and T cells differentiation at an early stage. In consequence, RAG deficient animals lack mature B and T lymphocytes (120, 121).

It was shown in several studies that arrival, localization and persistence of DCs in the spleen of $RAG^{-/-}$ mice are not particularly altered in comparison to WT mice (120, 141, 142). However, the numbers of non-erythroid cells in the spleen of $RAG^{-/-}$ mice is between five to nine times lower than numbers in the spleen of corresponding WT littermates (120, 141). The percentage of $RAG^{-/-}$ splenic cDCs is similar to the percentage of cDCs found in WT mice (Fig. 4.1A) (141, 142).

To investigate whether the absence of lymphocytes in the spleen of $RAG^{-/-}$ mice influences the resident cDCs, I first analyzed cDCs for different cell surface markers and co-stimulatory molecules. Comparable expression patterns in both WT and $RAG^{-/-}$ mice were revealed (Fig. 4.1B). Interestingly, I observed significant differences in the expression levels of MHC I and MHC II molecules. Such molecules were expressed at higher levels in $RAG^{-/-}$ cDCs. Although this might suggest different maturation status of $RAG^{-/-}$ and WT cDCs, the grossly equal levels of co-stimulatory molecules on cDCs in both mice argued to the contrary (Fig. 4.1B). In addition under inflammatory conditions, that normally result in full maturation of DCs the increase of expression of MHC molecules would be much more pronounced. Therefore, the maturation status of splenic cDCs from $RAG^{-/-}$ and WT mice may be considered similar.

4.2 Splenic cDCs from $RAG^{-/-}$ mice are impaired in presentation of soluble antigen

Since the major function of DCs is the presentation of antigen and activation of T cells, I decided to examine splenic cDCs from $RAG^{-/-}$ and WT mice in antigen presentation assay. First, I resorted to soluble OVA as a model antigen and OT I transgenic T cells as responder cells. Data depicted on Fig. 4.2 show that cDCs sorted from RAG deficient mice were highly impaired in cross-presentation of the intact

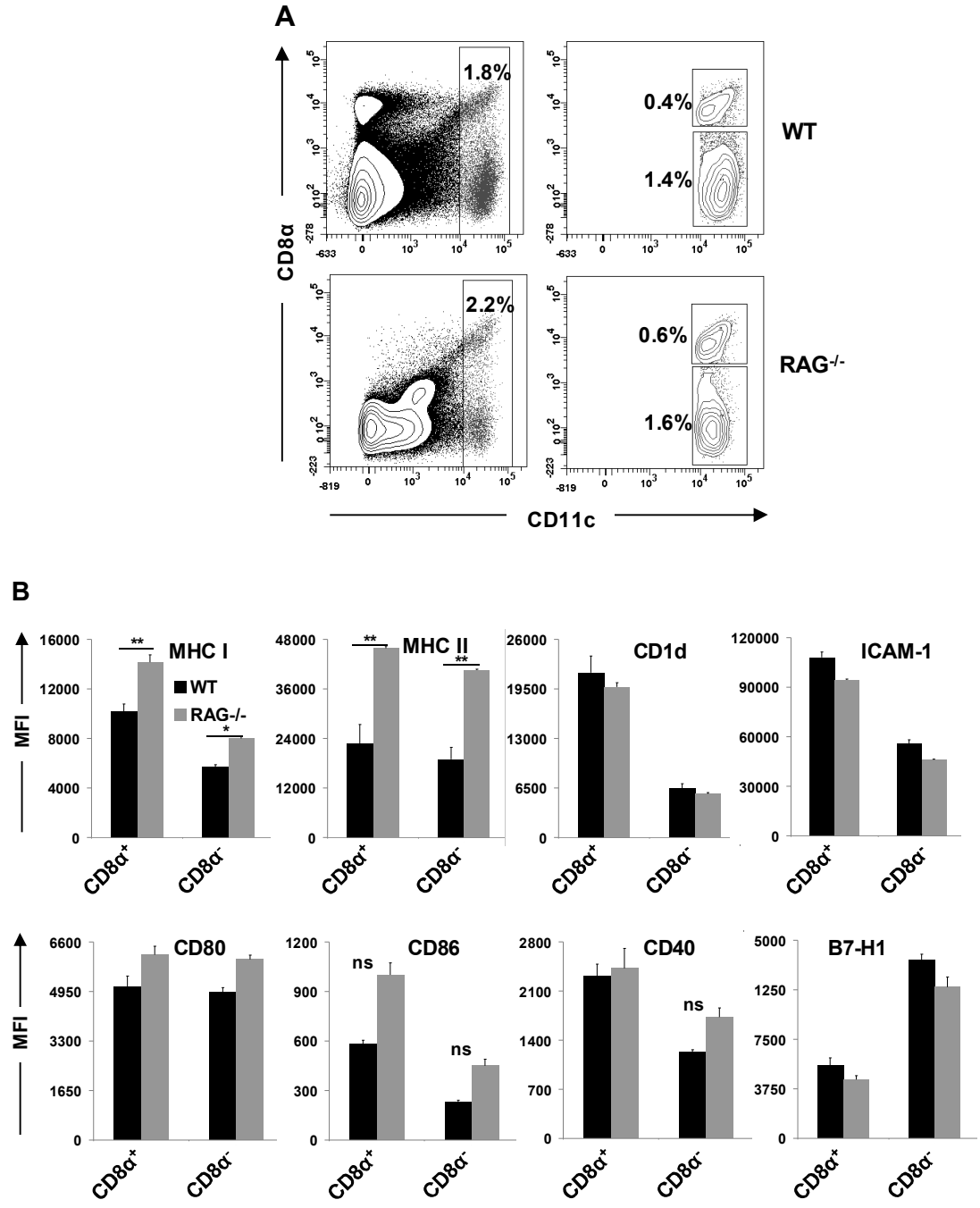


Figure 4.1 Similar percentage and surface phenotype of RAG$^{-/-}$ and WT splenic cDCs. (A) Splenocytes were isolated from WT and RAG deficient mice and stained with appropriate mAbs. Dot plots show similar percentage of CD11c^{hi} cDCs population in spleens of both mice. Two subpopulations of splenic cDCs were also similar in respect to their percentage in WT and RAG$^{-/-}$ mice. **(B)** Splenocytes of WT and RAG$^{-/-}$ mice were isolated, stained for indicated surface markers and analyzed by flow cytometry. Graphs show mean fluorescence intensity (MFI) values of each marker. Data are representative of at least 4-5 mice per group in six independent experiments. Statistical significance was determined using the paired Student's t test. * P<0.05; ** P<0.01; *** P<0.005, ns – not significant

OVA protein and to some extend even in presentation of "pre-processed" peptide ($OVA_{257-264}$).

Such properties of $RAG^{-/-}$ DCs were already described before (143), although in a different experimental model. My data substantiated that this might be a general characteristic of cDCs from RAG deficient mice.

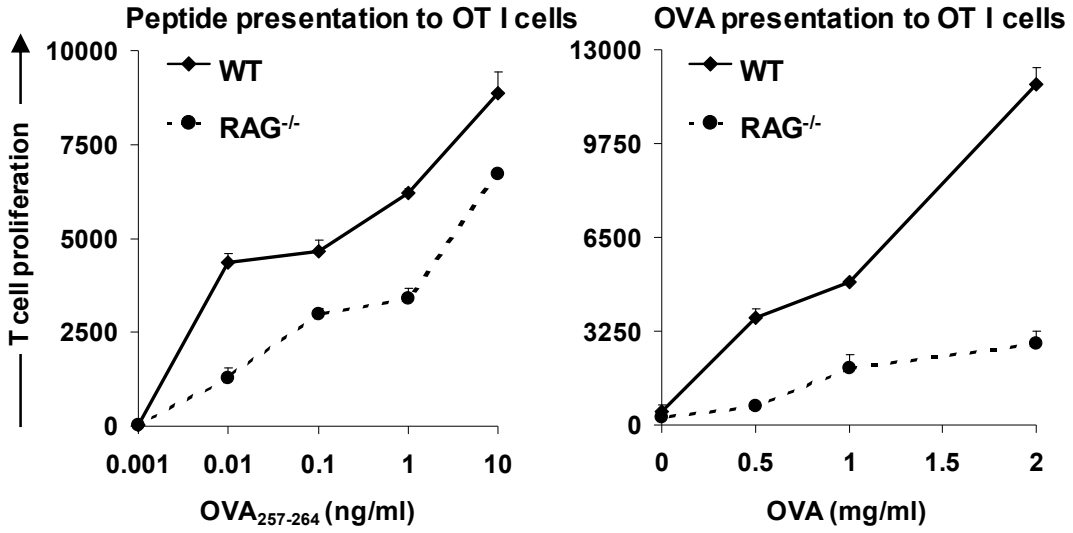


Figure 4.2 $RAG^{-/-}$ DCs are impaired in presentation of soluble OVA to OT I cells. Purified OT I transgenic T cells were labeled with CFSE and incubated for 1.5 days (peptide) or 2 days (OVA) with splenic cDCs ($CD11c^{hi}$, $CD11b^{+/-}$, $CD8\alpha^{+/-}$, $B220^{-}$) in ratio 10:1. First cDCs from WT and $RAG^{-/-}$ mice were loaded with $OVA_{257-264}$ peptide or whole OVA protein for 1h and further were washed intensively. The proliferative response of T cells was enumerated by flow cytometry. Data are representative of five independent experiments.

4.3 Transcriptional profile of cDCs from RAG deficient and WT mice

To investigate the molecular mechanism that could explain above observations, I decided to examine the transcriptional program of splenic cDCs from $RAG^{-/-}$ mice in comparison to cDCs from WT mice. Splenic cDCs from $RAG^{-/-}$ and WT mice were sorted and prepared RNA was analyzed by microarrays. I could distinguish three group of genes, which were differentially regulated between $RAG^{-/-}$ and WT cDCs: endosomal and lysosomal enzymes (endo-lysosomal enzymes), members of the C1q complement component and particular surface receptors and molecules (Fig. 4.3A- C). The level of up-regulation of genes in $RAG^{-/-}$ cDCs for endo-lysosomal enzymes varied between 45 and 10 times, for C1q complement component between 55 and 25

times and for surface receptors and molecules between 68 and 26 times. These three panels of genes should shed a new light on the character of RAG$^{-/-}$ cDCs and provide possible explanations for their impaired antigen presentation abilities.

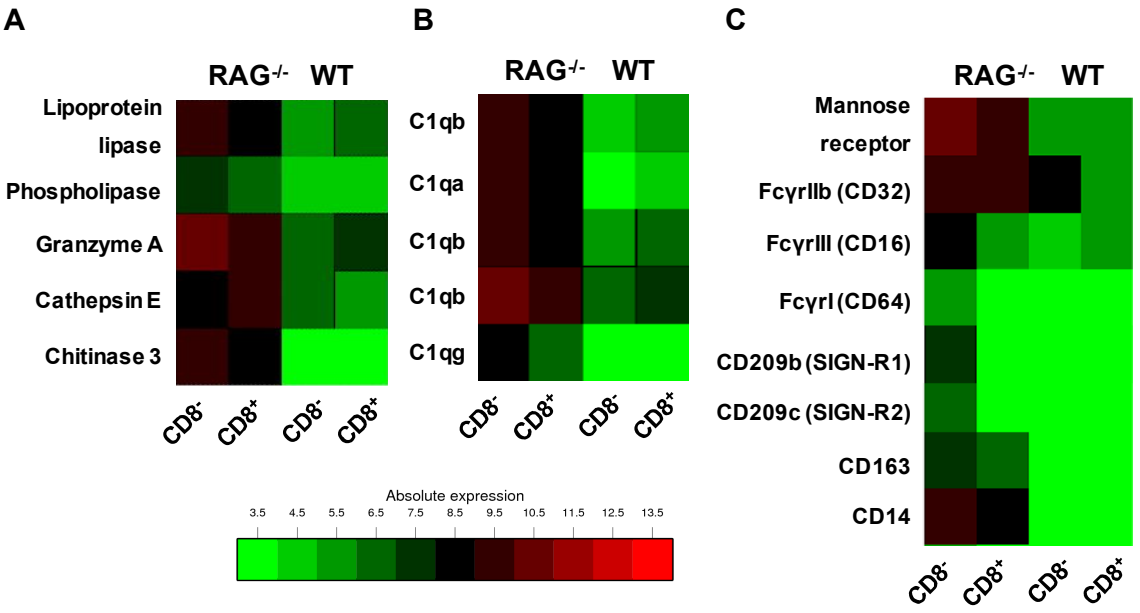


Figure 4.3 Transcriptional profile of RAG$^{-/-}$ cDCs show up-regulation of lysosomal and endosomal enzymes, C1q complement members, particular surface receptors and molecules in comparison to WT cDCs. (A-C) Affymetrix gene array analysis of splenic cDCs. mRNA was prepared from sorted splenic CD8α^{+}DCs and CD8α^{-} DCs from WT and RAG$^{-/-}$ mice. All samples were repeated twice with individually sorted cells and averaged**.**

The up-regulation of endo-lysosomal enzymes, which are responsible for antigen degradation, may suggest that RAG deficient cDCs exhibit more aggressive degradation properties. Once antigen is degraded excessively, APCs can no longer efficiently present it to T cells. This is reminiscent of the far more aggressive degradation of antigen by macrophages (Mφ) compared to DCs (144). On the other hand the up-regulated mannose receptor and other C-type lectin receptors, like SIGN-R1 and SIGN-R2 found in RAG$^{-/-}$ cDCs suggested a highly efficient up-take of soluble antigen and its deviation into the cross-presentation pathway (145). Nevertheless, even under these conditions high amounts of endo-lysosomal enzymes may degrade the antigen too fast to be loaded onto MHC I molecules and thus abrogating cross-presentation.

Beyond very well established role in initiation of the "classical pathway" of complement activation, members of C1q complement family are potent regulators of

DCs differentiation, activation and antigen presentation (146). They can opsonize apoptotic cells for DC phagocytosis and enhance the uptake of apoptotic cells without influence on surface expression of CD40 and CD86. Moreover, C1q was shown to have tolerogenic properties, since C1q deficient animals develop autoimmunity (146). This might also be connected with their inability to take up apoptotic cells. Since $RAG^{-/-}$ cDCs express higher levels of C1q complement members, they may be specialized in the presentation of particular antigens, like cell associated antigens as found in apoptotic cells.

Cell surface receptors for immunoglobulins (FcγR) are now well characterized and have been shown to play an important role in the immune system. They represent a paradigm for the balance of positive and negative signals, since they are generally present as receptor pairs that consist of activating and inhibitory molecules. FcγR are also well known for their role in setting the threshold for B cell activation. Moreover they were shown to regulate the maturation of DCs. Especially FcγRIIB was proposed to prevent spontaneous maturation of DCs, thereby promoting steady state tolerance (147, 148). Therefore, signaling through these receptors may represent an important issue in the regulation of $RAG^{-/-}$ cDCs function.

In addition to the higher expression of FcγR microarrays showed an up-regulation of CD163 (scavenger receptor) and CD14 (co-receptor for TLR4 and TLR2) by RAG deficient cDCs. Since all these molecules are known to be especially highly expressed by Mφ and monocytes this may suggest that the actual character of $RAG^{-/-}$ derived cDCs is similar to Mφ or monocytes.

4.4 Absence of T and B cells leads to enhanced protein degradation by splenic cDCs

The up-regulation of endo-lysosomal enzymes observed by microarrays suggested a more aggressive degradation of soluble antigen in RAG deficient cDCs. To confirm this I used two fluorescently labeled OVA proteins, OVA-Cy5 and DQ-OVA. OVA-Cy5 does not require processing to give fluorescence, therefore signal intensity in Cy5 channel indicate efficiency of Ag uptake by DCs. Opposite is true for DQ-OVA, it requires proteolytic degradation to give fluorescent signal, therefore for DQ-OVA signal intensity corresponds with Ag degradation. Indeed, as suggested by the expression level of surface receptors $RAG^{-/-}$ cDCs showed a slightly more efficient up-

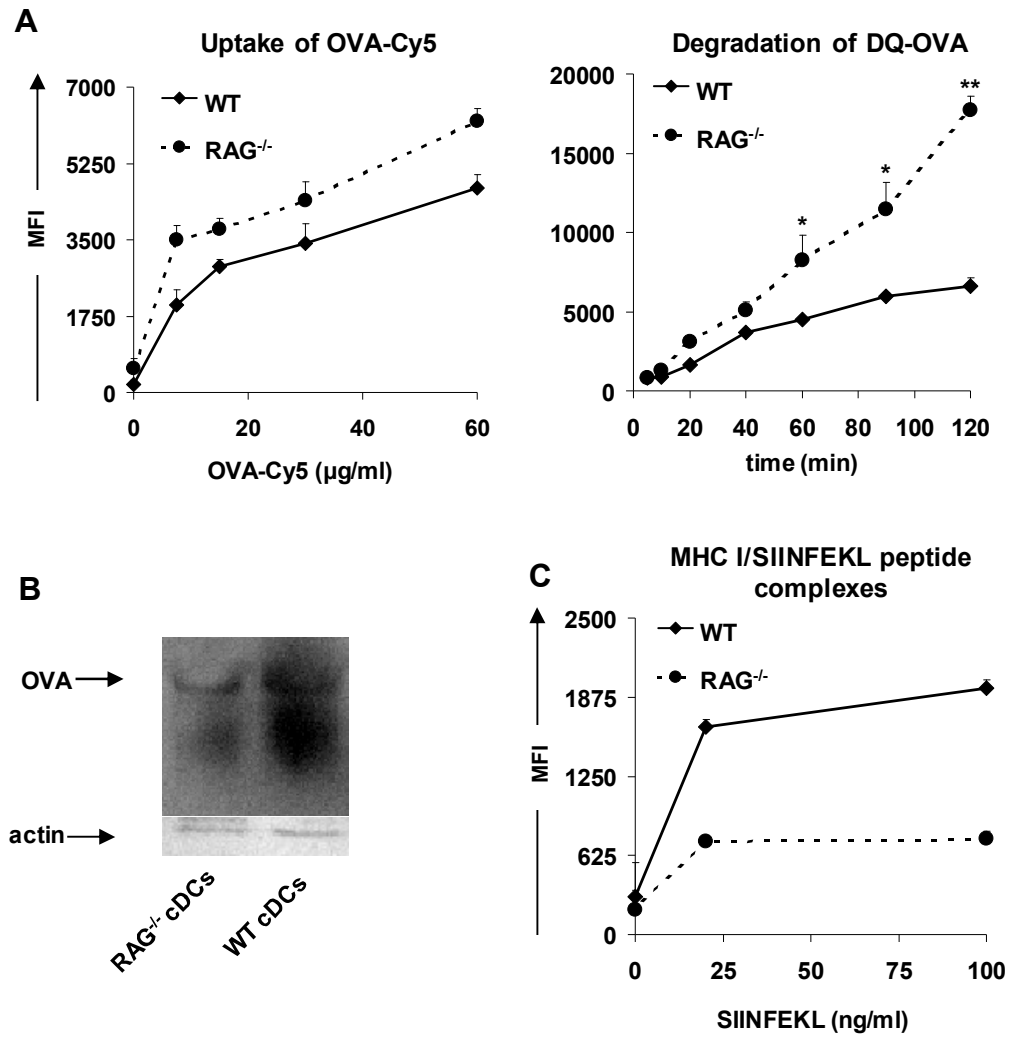


Figure 4.4 RAG deficient cDCs exhibit more aggressive degradation of soluble antigen in comparison to WT cDCs. (A) WT and $RAG^{-/-}$ splenic cDCs were sorted and incubated for 1h with indicated concentrations of OVA-Cy5 (left panel). WT and $RAG^{-/-}$ splenic cDCs were sorted and incubated with 62.5µg/ml of DQ-OVA for indicated time points (right panel). Further cells were washed intensively and analyzed by flow cytometry. **(B)** Sorted WT and $RAG^{-/-}$ cDCs were loaded with OVA-bio for 1h, washed intensively, lysed, separated in 10 % SDS-PAGE and transferred to a PVDF membrane. Further were incubated with appropriate Abs and developed with advanced ECL. **(C)** WT and $RAG^{-/-}$ were sorted, incubated with appropriate concentrations of OVA peptide SIINFEKL ($OVA_{257-264}$), washed carefully and stained with 25-D1.16 mAb. Number of MHC I/SIINFEKL complexes was assessed by flow cytometry. Data are representative of three independent experiments. Statistical significance was determined using the paired Student's t test. * $P<0.05$; ** $P<0.01$; *** $P<0.005$

take of OVA-Cy5 compared to WT cDCs. In addition, degradation of DQ-OVA was far more effective when $RAG^{-/-}$ cDCs were tested (Fig. 4.4A).

Aggressive degradation of soluble OVA by $RAG^{-/-}$ was confirmed by immunoblot. Sorted cDCs from WT and RAG deficient mice were loaded with biotinylated OVA (OVA-bio) and examined for the appearance of degradation products. After 1h OVA degradation products were clearly detectable in WT cDCs, where in $RAG^{-/-}$ cDCs they

were no longer present (Fig. 4.4B). Thus, in the absence of mature T and B lymphocytes, cDCs exhibit increased proteolytic activity for soluble proteins. As a consequence, lower numbers of MHC I/SIINFEKL peptide complexes are found on surface of $RAG^{-/-}$ cDCs compared to WT cDCs (Fig. 4.4C). Thus, despite the slightly higher amounts of antigen that is endocytosed by $RAG^{-/-}$ cDCs compared to WT cDCs, the rapid degradation hampers efficient antigen presentation.

4.5 Splenic cDCs from lymphopenic hosts can efficiently present cell associated antigens

Inefficient presentation of soluble antigen on the one hand and up-regulation of C1q complement components by splenic cDCs on the other hand (Fig. 4.3B and 4.5A) in the absence of mature T and B lymphocytes, suggested that such DCs may be prone to present peculiar antigens. Since C1q molecules are known to facilitate phagocytosis of apoptotic cells, I decided to test $RAG^{-/-}$ and WT cDCs for the presentation of antigens associated with apoptotic cells. To test this, I used the EG7-OVA cell line, a derivative the thymoma EL-4 transfected with OVA (127). EG7-OVA cells were UV-irradiated ($9mJ/cm^2$) to induce apoptosis, and further incubated with $RAG^{-/-}$ and WT cDCs. As responder cells OT I T cells were used. Interestingly, under these conditions T cell proliferation induced by $RAG^{-/-}$ cDCs was comparable to that induced by WT cDCs (Fig. 4.5B). This indicates, that indeed $RAG^{-/-}$ cDCs can efficiently phagocytose and present cell associated antigen derived from apoptotic cells.

Results from this experiment prompted me to investigate the overall content of apoptotic leukocytes in the spleen of RAG deficient mice. I performed staining using annexin V, which binds to the phosphoserine exposed on the cell surface at the early stage of apoptosis. Indeed, spleens of $RAG^{-/-}$ mice contained significantly more apoptotic cells than spleens of WT mice (Fig. 4.5C). This may explain why cDCs from $RAG^{-/-}$ mice are very efficient in presentation of cell associated antigens. Obviously, in their neighborhood an accumulation of apoptotic debris is found. Impaired clearance of apoptotic cells would lead to the pathological accumulation of necrotic corpses and in consequence to inflammation (149). Hence, an efficient phagocytosis of such debris is required to avoid an inflammatory response. Therefore, production of C1q complement components by cDCs may be initiated as mechanism to enhance the acquisition of apoptotic cells. These proteins were shown to opsonize apoptotic cells for DCs phagocytosis. Therefore, higher expression of C1q complement members in

RAG$^{-/-}$ cDCs as compared to WT may represent an adaptation for clearance of apoptotic cells present in the spleen.

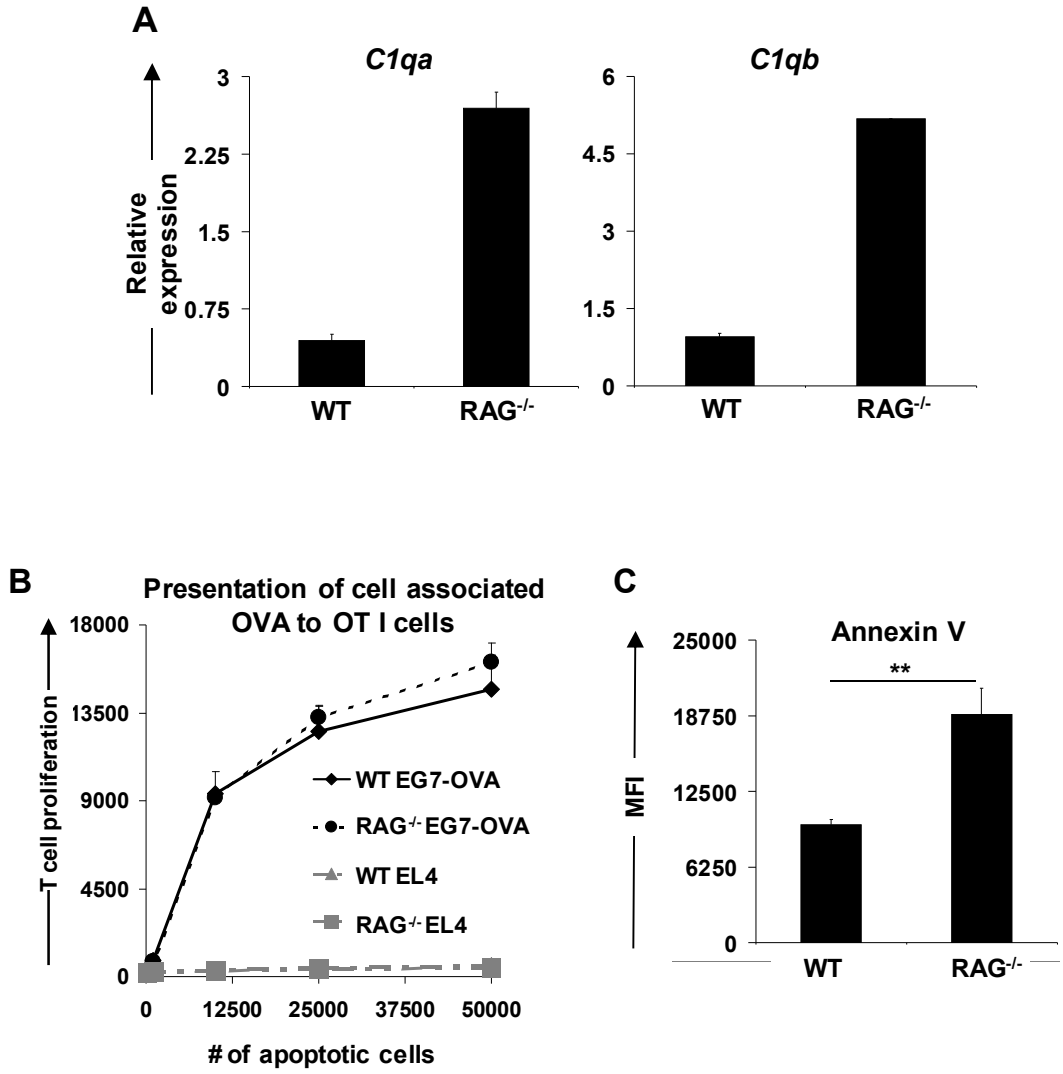


Figure 4.5 RAG$^{-/-}$ cDCs are specialized in presentation of cell associated antigens. (A) Quantitative real-time PCR (qRT-PCR) of RNA from splenic cDCs sorted from WT and RAG$^{-/-}$ mice for C1qa (left panel) and C1qb (right panel). **(B)** The purified OT I transgenic T cells were labeled with CFSE and incubated for 2 days with splenic cDCs (CD11c^{high}, CD11b$^{+/-}$, CD8α$^{+/-}$, B220^{-}) in ratio 10:1. cDCs from WT and RAG$^{-/-}$ mice were preloaded with UV-irradiated EG7-OVA cells for 1h. The proliferative response of T cells was enumerated by flow cytometry. **(C)** Splenocytes of WT and RAG deficient mice were isolated and stained with annexin V. Data were analyzed by flow cytometry. All data are representative of three independent experiments.

4.6 Impaired function of RAG deficient cDCs to present soluble antigen can be rescue by B cells

Next, I wanted to investigate whether the impaired capacity of cDCs from $RAG^{-/-}$ mice to present soluble antigen could be recovered. B cells were previously shown to regulate the function of DCs. For instance, immune responses to antigenic challenge differ in WT and B cell deficient mice (μMT). Although priming of T cells was not significantly different, the cytokine profile was clearly distinct (138). Unfortunately, this study did not particularly address antigen presentation by splenic cDCs. Another study (143) showed, that T cells are necessary for functional maturation of skin derived $RAG^{-/-}$ DCs, and that reconstitution of RAG deficient mice with T cells rescued ability of skin DCs to present soluble antigen. Despite of the latter data, I first addressed the question whether reconstitution of RAG deficient mice with B cells alone could recover the function of splenic cDCs.

To test this WT and $RAG^{-/-}$ mice were i.v. injected with bulk WT $CD19^{+}$ splenic B cells and after 3 weeks cDCs were sorted and their ability to cross-present soluble OVA to OT I T cells was tested. Isolated B cells were sufficient to restore the impaired function of $RAG^{-/-}$ cDCs (Fig. 4.6). Cross-presentation of soluble OVA by B cell reconstituted $RAG^{-/-}$ cDCs was as efficient as by WT cDCs. Therefore, B cells alone are sufficient to restore diminished ability to cross-present soluble antigen by RAG deficient splenic cDCs. Thus far, the impact of T cells could not be tested for reasons of time constrains. Therefore, a similar effect of T cells cannot be excluded.

4.7 Serum and soluble immunoglobulins can recover impaired function of cDCs from RAG deficient mice

The major function of B cells is to produce immunoglobulins. However, B cells might also be a source of cytokines. Microarray analysis had revealed the up-regulation of FcγR in $RAG^{-/-}$ cDCs. Therefore, binding of immunoglobulins to FcγR might most likely modify the function of DCs. In addition, it had been proposed before that engagement of FcγR on DCs triggers their maturation and induces efficient antigen presentation *via* both MHC I and MHC II (147).

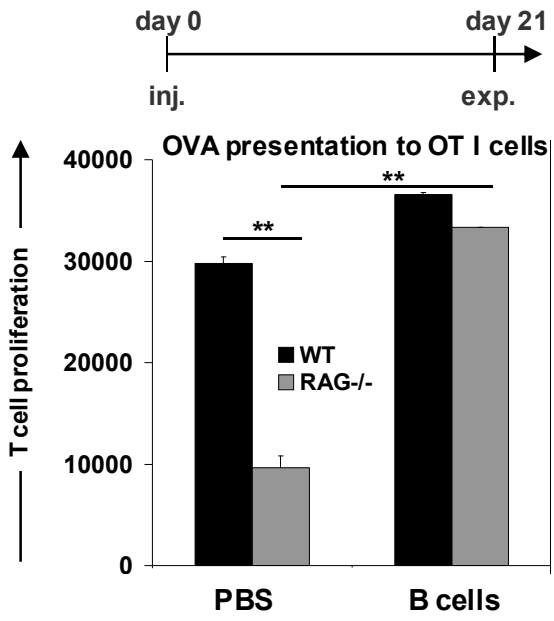
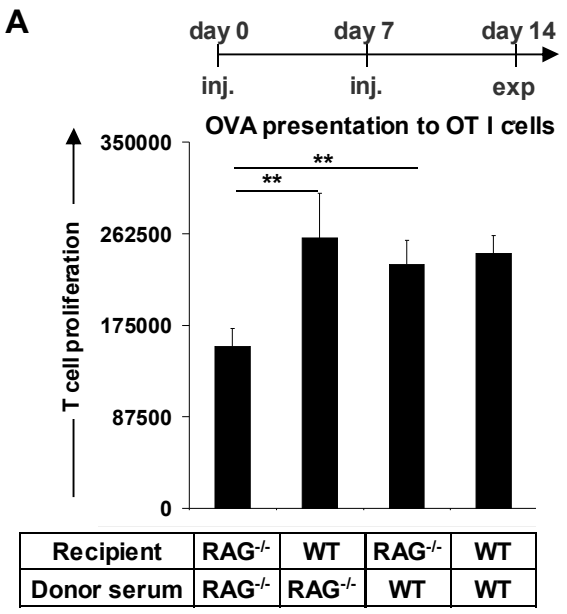


Figure 4.6 Reconstitution of RAG deficient mice with WT B cells leads to restoration of impaired function of RAG$^{-/-}$ cDCs. WT and RAG deficient mice were i.v. injected with sorted WT CD19^{+} B cells or PBS. After 21 days mice were sacrificed, splenic cDCs were isolated and tested for their ability to cross-present soluble OVA to OT I cells. At least 5 mice per group were used. Statistical significance was determined using the paired Student's t test. * $P<0.05$; ** $P<0.01$; *** $P<0.005$

Particularly FcγRIIB was proposed to prevent spontaneous maturation of DCs in steady state (148).

Therefore, I reconstituted RAG deficient mice with serum from WT mice. Data depicted on Fig. 4.7A show, that this treatment completely recovered the impaired ability of RAG$^{-/-}$ cDCs to present soluble OVA. This was consistent with the possibility that immunoglobulins are required for proper function of cDCs. Serum from RAG deficient mice did not recover this function excluding a contribution of activated complement or fibrinogen components.

To compellingly show the contribution of immunoglobulins to cDC maturation I administrated purified IgG and IgM to RAG$^{-/-}$ mice. This experiment confirmed that the serum effect was due to immunoglobulins. DCs from IgG or IgM injected RAG$^{-/-}$ mice were as efficient in cross presentation of soluble OVA to OT I cells as WT cDCs (Fig. 4.7B). Therefore, in extension to published data (143) I could show, that soluble immunolglobulins alone are sufficient to recover the impaired antigen presentation capacity of cDCs isolated from RAG deficient mice.



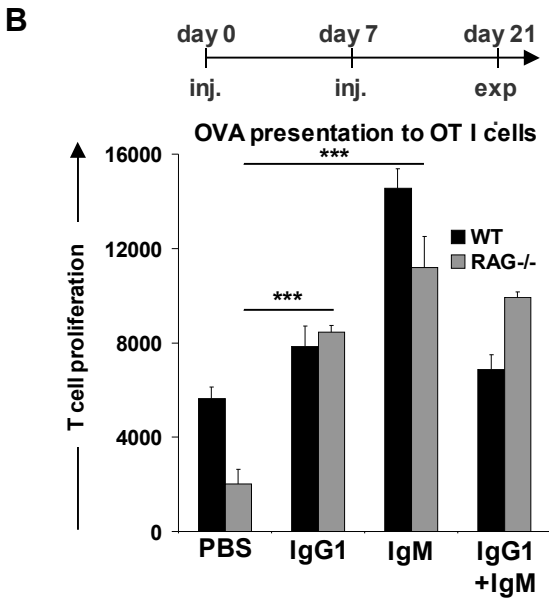


Figure 4.7 Reconstitution of RAG deficient mice with soluble immunolglobulins recovered function of cDCs to cross-present soluble OVA. (A) WT and RAG$^{-/-}$ recipient mice were i.v. injected on day 0 and day 7 with 50µl of donor serum. After 14 days splenic cDCs were isolated from recipient mice and tested in antigen presentation assay. **(B)** WT and RAG$^{-/-}$ recipient mice were i.v. injected on day 0 and day 7 with 75µg/ml of IgG, IgM alone or together. After 21 days splenic cDCs were isolated from recipient mice and tested in antigen presentation assay. Data are representative of two independent experiments. Statistical significance was determined using the paired Student's t test. * P<0.05; ** P<0.01; *** P<0.005

CHAPTER V

RESULTS PART III

DIFFERENTIATION OF NKT CELLS INDUCED BY MARGINAL ZONE B CELLS COMPARED TO CONVENTIONAL SPLENIC DENDRITIC CELLS

The marginal zone (MZ) of the spleen is a unique structure, found only in this organ. The localization and architecture of MZ favors contact with blood borne antigens and pathogens. It is characterized by a peculiar composition of leukocytes and lymphocytes. Besides macrophages and cDCs, NKT cells are found here. In addition, marginal zone (MZ) B cells home to this structure. MZ B cells are claimed to be maintained in a pre-activated state. This allows them to respond rapidly to pathogens by antibody production (62-64).

Importantly, amongst all murine APCs, MZ B cells were found to express the highest levels of CD1d, the non-classical MHC I molecule that is responsible for presentation of lipid and glycolipid antigens. CD1d/lipid complexes are recognized by NKT cells that are known to rapidly secrete cytokines such as IL-4 and IFN-γ. This suggests a decisive regulatory function during the initial phase of an immune response. Due to the high levels of CD1d and the co-localization of both cell types, I postulated that MZ B cells may be efficient activators of NKT cells.

Therefore, I used MZ B cells and splenic cDCs sensitized with α-galactosylceramide as APCs for NKT cells stimulation. Both MZ B cells and cDCs proved to be highly efficient in priming of NKT cells and to induce their robust proliferation *in vitro*. Interestingly, MZ B cells exclusively induced production of IL-4 by NKT cells whereas cDCs induced robust production of IFN-γ.

In addition, I could demonstrate that proliferation of NKT cells and IL-4 production activated by MZ B cells was dependent on ICOS/ICOSL interactions. Taken together, these data suggest that both MZ B cells as well as cDC act as professional APCs for NKT cells. Notably, the nature of APC appears to be critical for the polarization of the immune response: MZ B-cell-primed NKT cells to induce a cytokine milieu that would foster a T_H2 response, whereas cDC-primed NKT cells rather favor a T_H1 response.

5.1 Marginal zone B cells express high levels of CD1d and co-stimulatory molecules

To investigate the cell stimulatory capacity of MZ B cells, I first decided to carefully characterize the phenotype of MZ B cells in comparison to other splenic B cells, like B2 and B1a cells and in addition to splenic cDCs. Fig. 5.1A shows the gating strategy for MZ B cells, splenic B cells and cDCs that was used for cell surface analysis as well as for later cell sorting.

MZ B cells are defined as $CD19^{+}CD11c^{-}CD23^{-}$and $CD21/35^{+}$ (62-64). For comparison $CD11c^{hi}CD19^{-}$ cDCs were employed, which are known to be very efficient stimulators of T and NKT cells (Fig. 5.1B-C). Results revealed that MZ B cells express very high levels of the CD1d molecule. In fact expression was 7 to 8 time higher compared to cDCs. This feature of MZ B cells had been observed before (150-152). In contrast, B1 cells express CD1d only 2-3 times higher compared to cDC while expression of this molecule by B2 B cells was comparable to that found on cDCs. The high level of CD1d on MZ B cells confirmed their potential importance in activation of NKT cells.

5.2 Marginal zone B cells can efficiently stimulate proliferation of NKT cells *in vitro*

To test this, three types of splenic B cells, B1, B2 and MZ B, as well as cDC were purified by cell sorting and tested for their ability to present glycolipids (Scheme 5.1). They were sensitized with different concentrations of α-galactosylceramide (αGalCer). NKT cells ($lin^{-}Dx5^{-}NK1.1^{int}$) were isolated from the spleen of Vα14 transgenic mice, which contain about 3% of such cells. Sensitized APCs were co-cultured at a ratio of 1:10 with CFSE-labeled NKT cells and proliferation of NKT cells was measured 2.5 days later by flow cytometry (Fig. 5.2A and B). Interestingly, MZ B cells are very potent activators of NKT cell expansion, comparable to cDCs although at the highest tested concentration of αGalCer, MZ B cells were roughly two times more efficient in priming NKT cells compared to cDCs (Fig. 5.2B). Both, MZ B cells and cDCs, when sensitized with lower concentrations of αGalCer (0.2μg/ml) are still able to activate NKT cells efficiently.

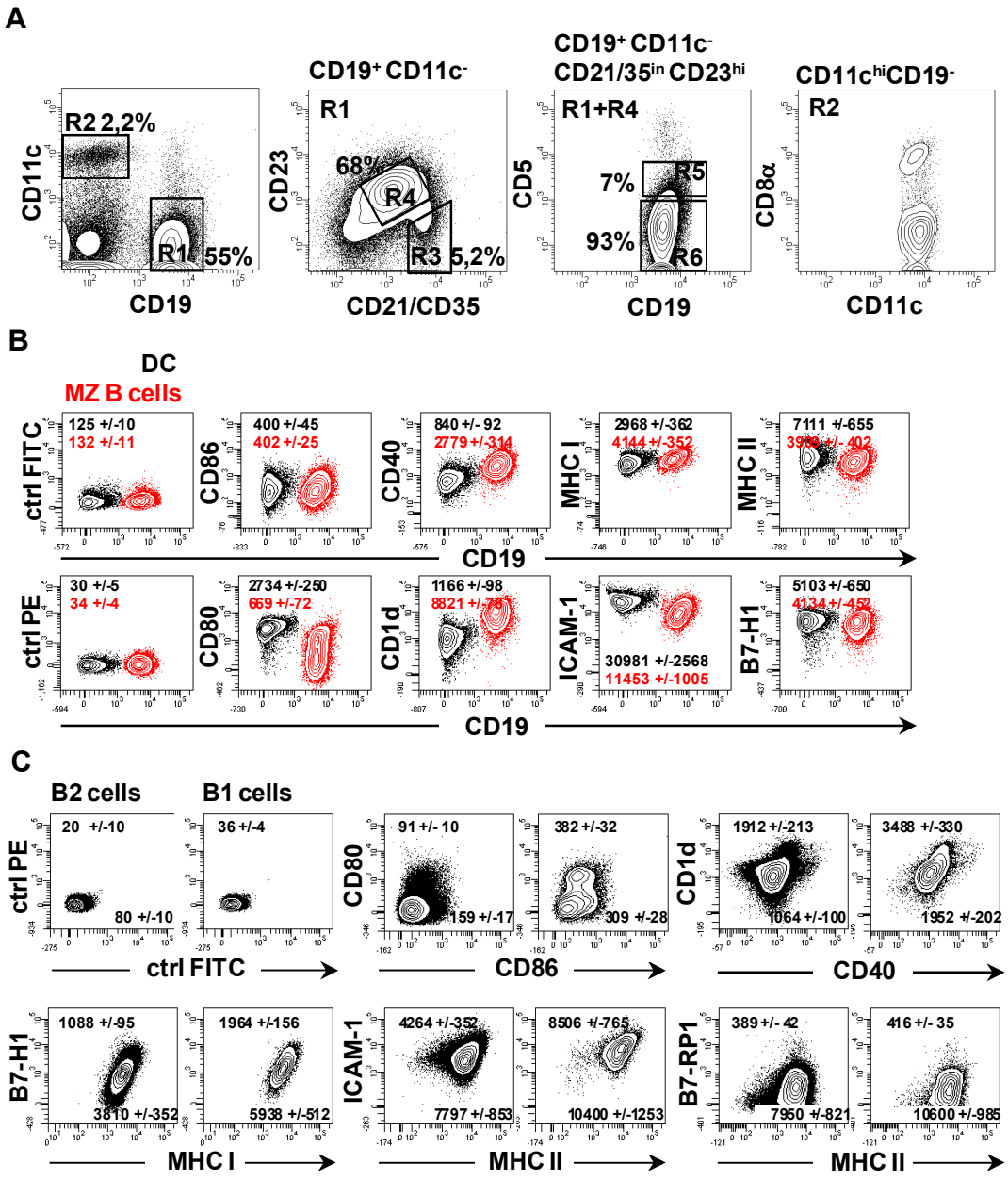
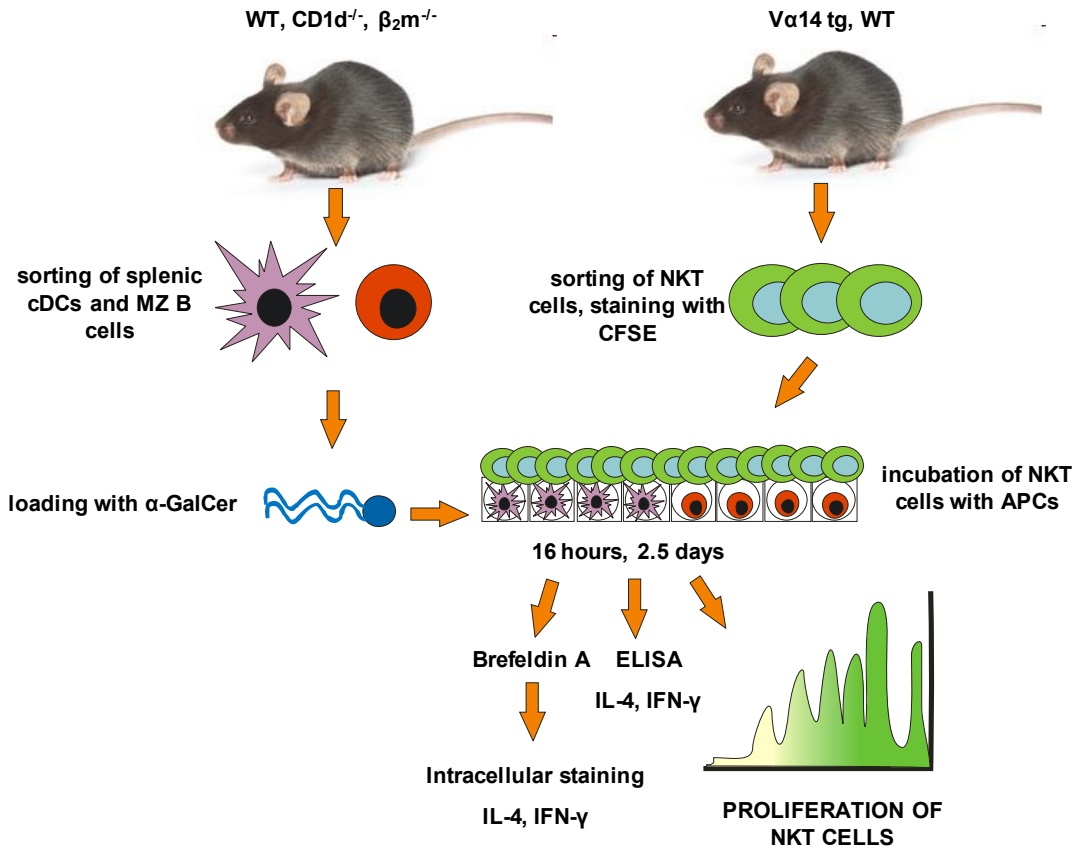


Figure 5.1 Sorting strategy and analysis of cell surface phenotype of different splenic APCs. (A) Splenocytes of WT mice were isolated, stained and sorted according to indicated gates. Cells were analyzed for CD11c and CD19 expression, $CD19^{+}$ $CD11c^{-}$ cells were analyzed for CD21/CD35, CD23 and CD5. MZ B cells are $CD21/35^{hi}CD23^{-/lo}$, B1 cells $CD21/35^{lo}CD23^{+}CD5^{lo}$ and B2 cells are $CD21/35^{lo}CD23^{+}CD5^{-}$. Splenic conventional DCs were $CD11c^{hi}CD8\alpha^{+/-}$. Cell populations were sorted with purity of 95% to 99%**. (B)** Comparison of co-stimulatory markers on MZ B cells and cDCs. MZ B cells indicate the highest expression of CD40 and CD1d surface proteins among all APCs found in steady state spleen. **(C)** Expression of DC associated co-stimulatory molecules by B2 and B1 cells. Values in dot-plots indicate mean fluorescent intensity (MFI) of analyzed markers. Representative staining for five independent experiments are shown.

In contrast splenic follicular B cells, B2 and B1 were not able to induce NKT cell proliferation, albeit levels of CD1d and co-stimulatory molecules were similar. This experiment shows that indeed MZ B cells alone are able to induce robust NKT cell proliferation *in vitro*.

High expression of CD40 molecules on both MZ B cells and cDCs suggested that activation of such APCs *via* CD40-CD40L interaction may significantly increase their stimulatory capacity during co-culture with NKT cells. To test whether CD40 signaling would increase the stimulatory capacity of such APCs, mice were treated with an agonistic anti-CD40 antibody for four hours. The same splenic cell populations were isolated and tested for stimulation of NKT cells as described before. However, stimulation of MZ B and cDCs with anti-CD40 *in vivo* increased their efficiency to prime NKT cell only mariginally (Fig. 5.2C). B1 and B2 cells still remained unable to stimulate NKT cell proliferation despite the treatment. Thus, the licensing by CD40L, that is observed for other APCs when priming T cells does not apply here.



Scheme 5.1 Schematic representation of the experimental strategy employed for *in vitro* NKT cells activation and proliferation assays.

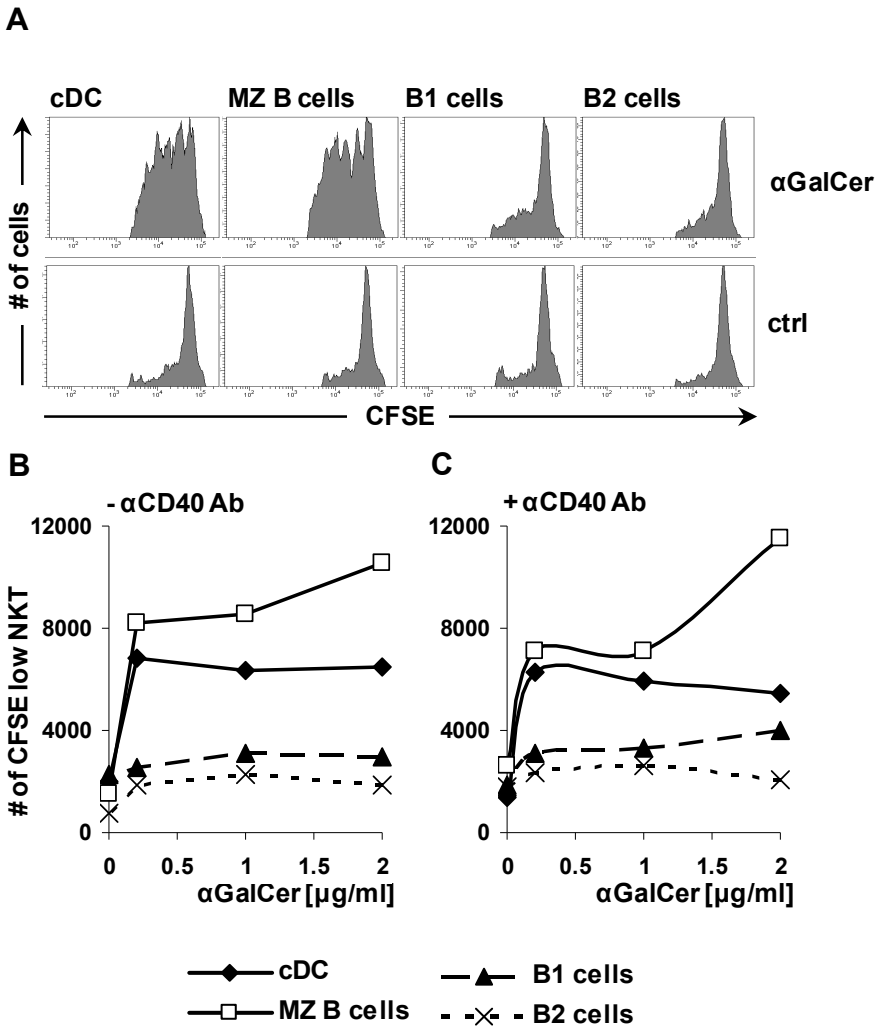


Figure 5.2 MZ B cells can efficiently present lipids to NKT cells *in vitro*. **(A)** Purified APCs were loaded with αGalCer (1µg/ml), washed and co-cultured with NKT cells for 2.5 days in ratio 1:10. Proliferation of NKT cells was determined in CFSE dilution assay. **(B)** Antigen concentration dependence of stimulation of NKT cells. **(C)** APCs were purified from mice treated with anti-CD40 Ab (150µg/mouse), loaded with αGalCer and co-cultured with NKT cells. Conditions of the test were like in A Data are representative of three independent experiments.

5.3 NKT cells produce IL-4 upon activation by MZ B cells

NKT cells are known to quickly secrete cytokines upon stimulation and may produce cytokines associated either with a T_H1 or T_H2 immune response. Therefore, their role during the immune response might be to drive T cells into a particular direction. The APC on which the NKT cells encounter the antigen might be decisive in this reaction. Since cDCs and MZ B cells exhibited a similar ability to prime NKT cell proliferation,

the question arose whether a similar cytokine profile would be elicited after the stimulation by the different APCs. As shown in Fig. 5.3, cDCs primed with αGalCer are able to efficiently stimulate NKT cells to produce IFN-γ. Intracellular staining indicated that around 14% of NKT cells produced IFN-γ after 16h (Fig. 5.3A). The percentage of NKT cell producing IFN-γ increased even up to 50% after 2.5 days of stimulation (Fig. 5.3B). cDCs were also able to induce production of IL-4. However, the percentage of positive cells was clearly lower – around 5% after 16h and 15% after 2.5 day of co-culture. Most of such NKT cells produced IFN-γ and IL-4 at the same time. None of them was triggered to produced IL-17 or IL-10 (data not shown).

In contrast, MZ B cells loaded with αGalCer were not able to induce the substantial production of IFN-γ by NKT cells, observed for cDC (Fig. 5.3A, B). Only 6.7% and 4.8% of NKT cells produce IFN-γ after 16 hours or 2.5 days of stimulation, respectively. However, MZ B cells appear to be more prone to induce IL-4 in NKT cells. After 16 hours of stimulation 3.5% and after 2.5 days 8.3% of NKT cells produce IL-4. Double producers were scarce under these conditions.

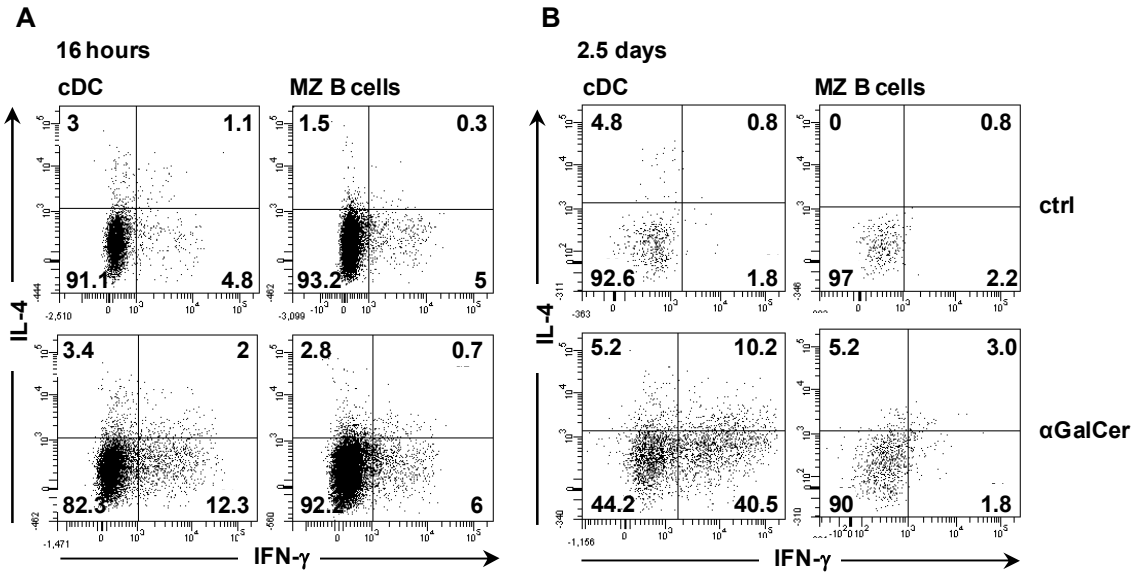


Figure 5.3 NKT cells produce IFN-γ only after stimulation with cDCs. Production of cytokines by NKT cells. cDC or MZ B cells were loaded with 1μg/ml of αGalCer, washed and co-cultured with NKT cells in ratio 1:1 for 16h **(A)** and 2.5 days **(B)**. Next, cells were incubated with Brefeldin A for 4 h, fixed, permeabilized and stained intracellularly for IFN-γ and IL-4. Data are representative of four independent experiments**.**

This bias became more obvious when supernatants of such cultures were tested by ELISA (Fig. 5.4A and B). cDCs upon αGalCer sensitization induce massive secretion of IFN-γ by NKT cells, while MZ B cells failed to do so (Fig. 5.4A). In contrast, MZ B cells sensitized with αGalCer efficiently induced IL-4 secretion by NKT cells at 16h (Fig. 5.4B). After 2.5 days, both MZ B cells and cDCs loaded with αGalCer stimulated NKT cells to produce IL-4 to the same extend. None of the other APC populations sensitized with αGalCer was able to stimulate NKT cells efficiently (Fig. 5.4 and data not shown).

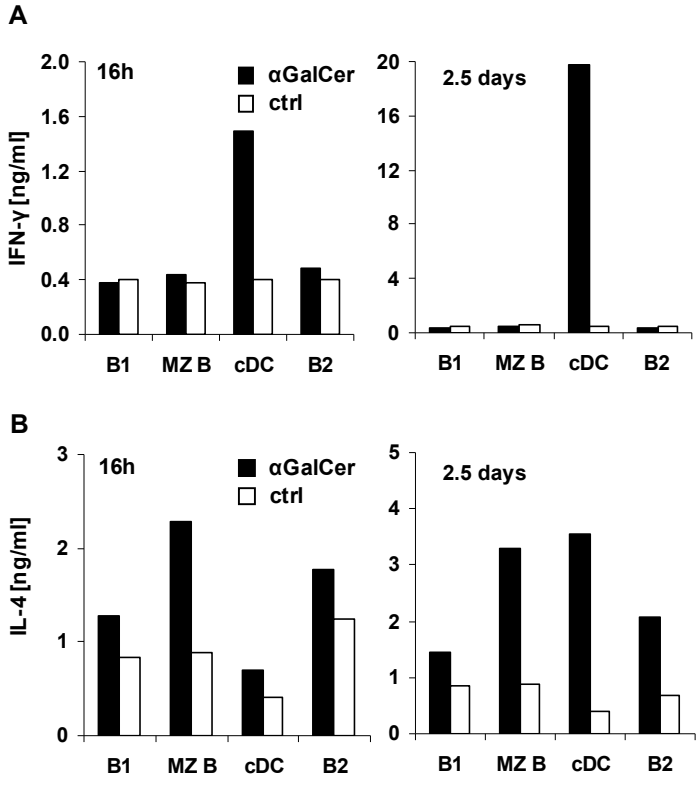


Figure 5.4 Efficient cytokines production by NKT cells requires presentation of glycolipid by splenic APCs. Three populations of splenic APCs: B2, MZ B cells and cDCs were purified as previously described. Cells were sensitized with 1μg/ml of αGalCer and incubated for 2.5 days with CFSE labeled NKT cells in ratio 1:2. After 16 and 2.5 days incubation of APCs with NKT cells, supernatants were collected and concentration of **(A)** IFN-γ and **(B)** IL-4 was determined in ELISA. Data are representative of three independent experiments.

5.4 Proliferation of NKT cells is CD1d dependent

In contrast to conventional αβ T cells, NKT cells can proliferate without stimulation *via* the TCR. For instance, cDCs stimulated by TLRs agonists can produce cytokines, mainly IL-12 and IL-18, which in turn are able to stimulate NKT cells to proliferate as

well as to produce cytokines. To test whether proliferation of NKT cells and synthesis of IFN-γ or IL-4 observed here depends on CD1d/TCR interaction or is driven by the IL-12, the following experiment was performed. cDCs and MZ B cells were sorted from the spleens of WT and $CD1d^{-/-}$ or $\beta_2m^{-/-}$ mice, which are unable to express CD1d molecules. Purified APCs were sensitized αGalCer and co-cultured with CFSE labeled NKT cells at a ratio of 1:2. In addition, to test whether IL-12 is involved in NKT cell proliferation and cytokine production, anti-IL-12 blocking Ab was added to some cultures. Proliferation was measured 2.5 days later (Fig. 5.5A).

When cDCs of $CD1d^{-/-}$ or $\beta_2m^{-/-}$ mice were used activation of NKT cells depends on secreted cytokines only. In this case NKT, cell proliferation was completely blocked by addition of anti-IL-12 Ab. By extrapolation, also under normal conditions proliferation of NKT cells induced by cDC might be partially dependent on IL-12 (Fig. 5.5A).

In contrast to cDCs, proliferation of NKT cells driven by MZ B cells appeared to be completely CD1d dependent. NKT cell primed by MZ B from knockout mice almost completely failed to proliferate. Since the MZ B cells cannot produce IL-12, addition of anti-IL-12 did not change these results as expected (Fig. 5.5A).

Analysis of cytokine production (Fig. 5.5B-C) led to several conclusions. After sensitization of cDCs by αGalCer, CD1d signaling is irreplaceable and leads to strong reduction of IFN-γ production. Blocking of IL-12 has strong impact on cytokine production by NKT cells. Absence of both CD1d and IL-12 leads to complete abrogation of IFN-γ synthesis. In contrast, MZ B cells can induce IL-4 production in NKT cells even in the absence of CD1d, although this is diminished (Fig. 5.5C).

5.5 Marginal zone B cells express higher levels of GITRL and ICOSL compared to cDCs

It was important to find the surface molecules that are responsible for an interaction between NKT cells and MZ B cells and their unique stimulatory capacity.

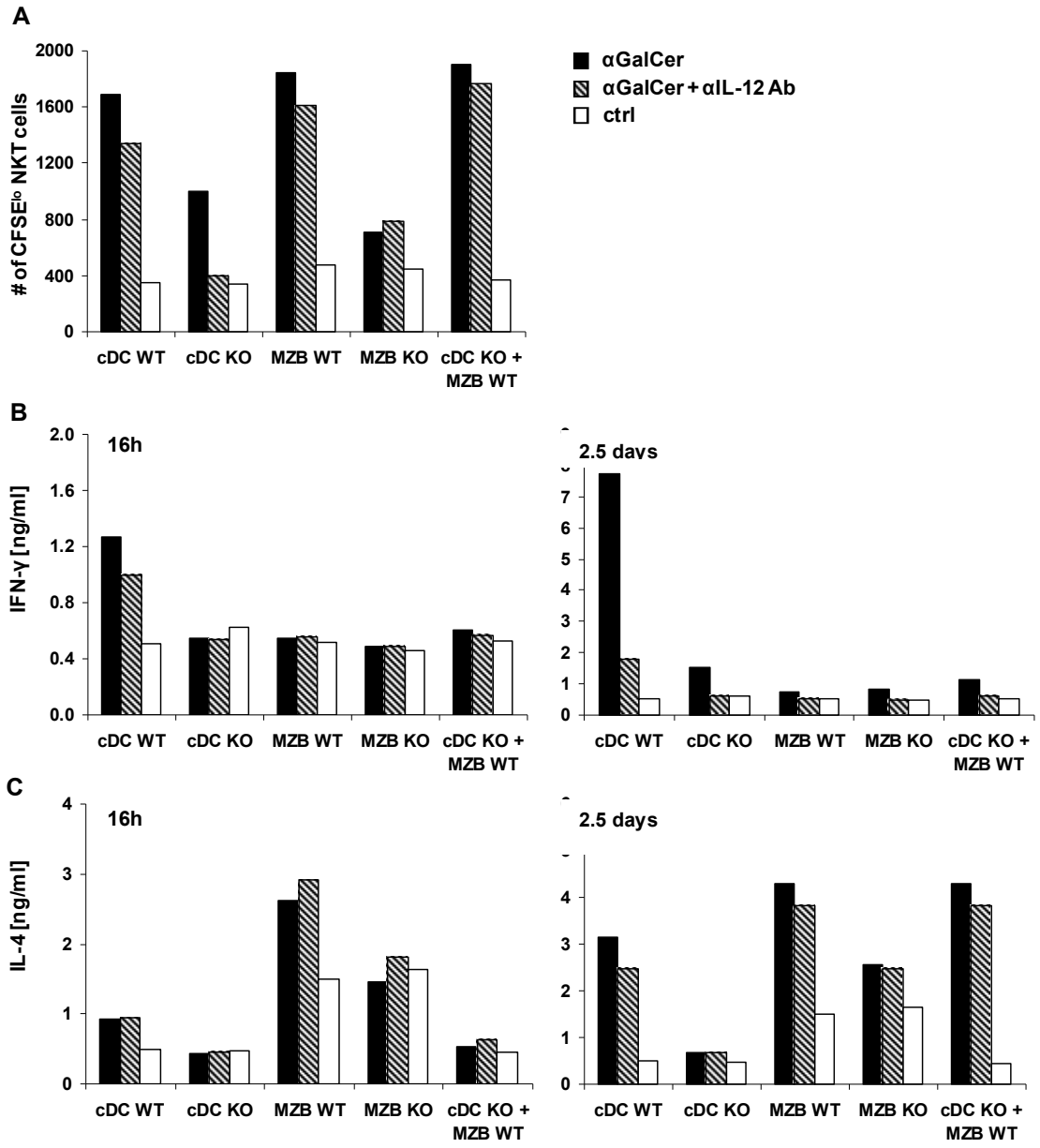


Figure 5.5 Proliferation of NKT cells is CD1d dependent. MZ B cells and cDCs were isolated from WT and CD1d$^{-/-}$ or β_2m$^{-/-}$ (KO). Cells were loaded with 1μg/ml of αGalCer and incubated for 2.5 days with CFSE labeled NKT cells in ratio 1:2. **(A)** Number of divided (CFSE^{lo}) NKT cells was determined in FACS. (B-C) MZ B cells and cDCs form WT and CD1d$^{-/-}$ or β_2m$^{-/-}$ mice were prepared as in A. After 16 and 72h hours of incubation with NKT cells, supernatants were collected and concentration of **(B)** IFN-γ and **(C)** IL-4 was determined in ELISA. Data are representative of three independent experiments.

I first decided to analyze the expression levels of ligands and their receptors, which are known to be involved in interplay between conventional APCs and NKT cells. Hence, I compared MZ B cells and cDCs for the expression of OX40L, CD27, GITRL and ICOSL (153-156). This analysis revealed that two ligands – GITRL and ICOSL were expressed significantly higher on the surface of MZ B cells in comparison to cDCs

(Fig. 5.6). GITR has been previously shown to be essential for activation of NKT cells (156), while ICOSL can stimulate $CD4^+$ T cells to secrete IL-4 rather than IFN-γ (155, 157).Therefore they represent potential candidates, specific for MZ B cells to stimulate expansion of NKT cells and their early IL-4 production.

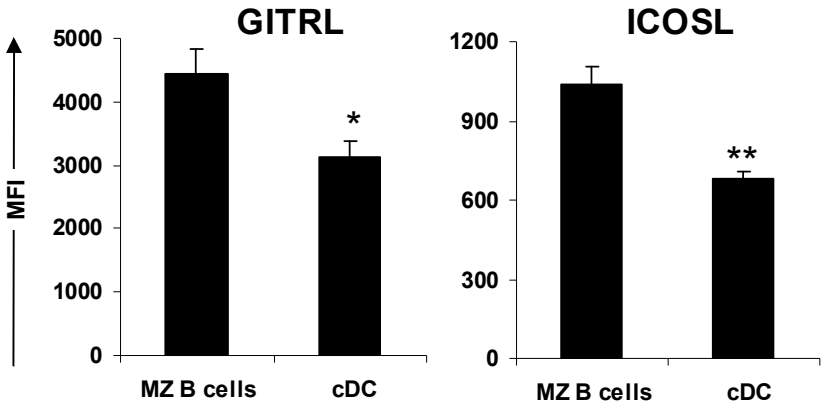


Figure 5.6 MZ B cell express higher surface levels of GITRL and ICOSL in comparison to cDCs. Splenic cDCs and MZ B cells were isolated and stained for GITRL and ICOSL with appropriate mAbs. Graphs show mean fluorescence intensity values (MFI) for indicated molecules. Picture is representative of three independent experiments.

5.6 Marginal zone B cells involve ICOS/ICOSL interactions to induce NKT cells proliferation and IL-4 production

To examine whether GITRL, ICOSL or both are involved in activation and differentiation of NKT cells when MZ B cells or cDCs were used as APCs blocking antibodies were employed. Data presented on Fig. 5.7A, and B shows, that proliferation of NKT cells and IL-4 production was not inhibited when a blocking anti-GITR antibody was added for both MZ B cells and cDCs. However, when cDCs were examined for their capacity to induce IFN-γ in NKT cells, anti-GITR Ab strongly diminished the response (Fig. 5.7C). Thus, MZ B cells do not use GITR/GITRL interaction to drive NKT cells expansion and IL-4 production while, cDCs require this interaction to stimulate IFN-γ production.

Then the second candidate, ICOSL was examined. Interestingly, in this case I found that proliferation of NKT cells, was highly impaired when they were stimulated by MZ B cells in presence of anti-ICOSL Ab (Fig. 5.7D). Similarly, IL-4 production by

NKT cells activated by MZ B cells was almost completely blocked (Fig. 5.7E). In contrast, IFN-γ production triggered by cDCs was not affected by this Ab (Fig. 5.7F). Thus, the dichotomy observed for cytokine production is reflected by a dichotomy of the co-stimulatory molecule involved in triggering the NKT cell response.

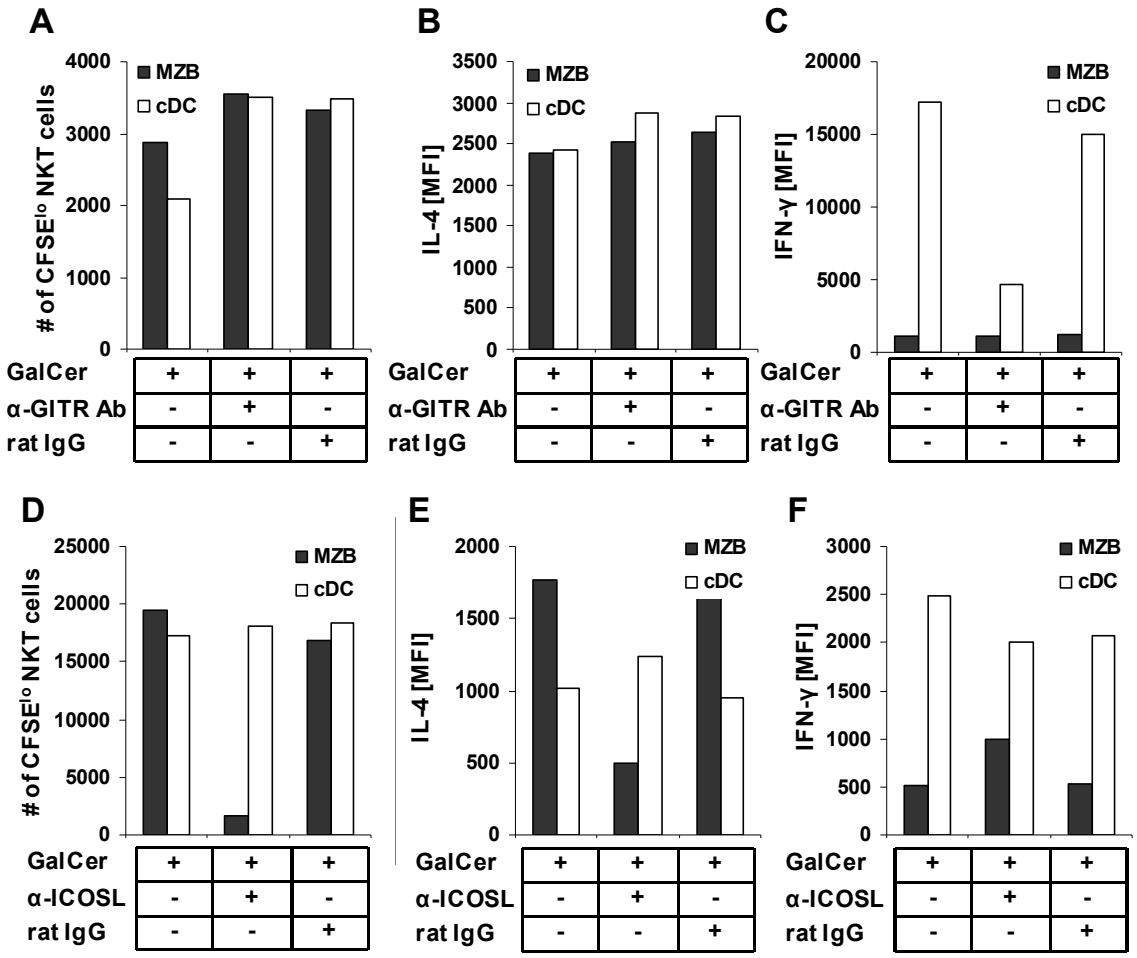
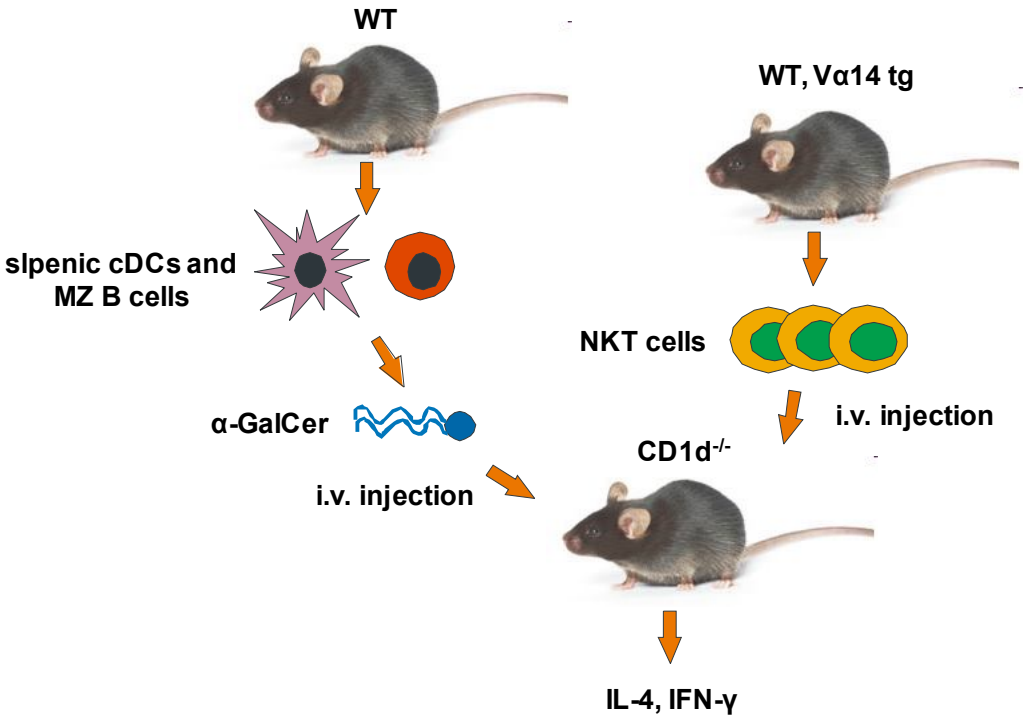


Figure 5.7 MZ B cells require ICOS/ICOSL interaction to stimulate NKT cells proliferation and IL-4 production. (A-C) MZ B cells do not involve GITR/GITRL interaction to stimulate NKT cell proliferation and IL-4 production but this interaction is required for IFN-γ production when cDCs are used as APCs. MZ B cells and cDCs were isolated and sorted as before, further were loaded with αGalCer (1μg/ml) and incubated with sorted NKT cells in ratio 1:1 for 2.5 days in the presence of blocking anti-GITR Ab. **(D-F)** MZ B cells require ICOS/ICOSL interaction to stimulate NKT cell proliferation and IL-4 production. Assay conditions as described above, cells were incubated in the presence of blocking anti-ICOSL Ab. Data are representative of three independent experiments.

5.7 Marginal zone B cells can efficiently present lipids to NKT cells *in vivo*

The final important question was, whether the cellular interactions demonstrated above have some *in vivo* relevance. To demonstrate this, sorted MZ B cells and cDCs from spleens of WT mice were loaded *ex vivo* with αGalCer to be tested *in vivo* for their NKT cell stimulatory capacity. However, MZ B cells are known to be able to transfer antigen to other immune cells, like DCs (66). Therefore, such sensitized APCs were transferred into CD1d deficient mice. The APCs of CD1d deficient mice are not able to present lipid antigens by themselves. Unfortunately, NKT cells cannot develop in the absence of CD1d. To compensate this defect, 2h before the assay an APC-free population of NKT cells was injected i.v. into the recipient mice. Subsequently, the sorted, antigen loaded APCs were administered. After 16h, spleens of recipient mice were removed, and splenocytes were analyzed for intracellular IL-4 and IFN-γ (Scheme 5.2).

Results from this experiment confirmed that MZ B cells alone are indeed very potent stimulators of NKT cells. In addition, they are able to induce production of IL-4 exclusively (1.6% positive cells) (Fig. 5.8). I could confirm, that cDC can induce a robust production of IFN-γ (11.3% positive cells) and only a moderate production of IL-4 by NKT cells (2.5% of positive cells). This experiment clearly shows that the different stimulatory capacity of MZ B cells and cDC are not limited to *in vitro* conditions. Also *in vivo*, MZ B cells appear as APCs that stimulate NKT to secrete moderate amounts of IL-4 and in consequence provide a cytokine milieu that favors T_H2 responses. In contrast, cDCs are very potent NKT cell activators. Upon such stimulation, NKT cells secrete mostly IFN-γ, and therefore promote a T_H1 immune response.



Scheme 5.2 *In vivo* NKT cells activation by sensitized MZ B cells or cDCs.

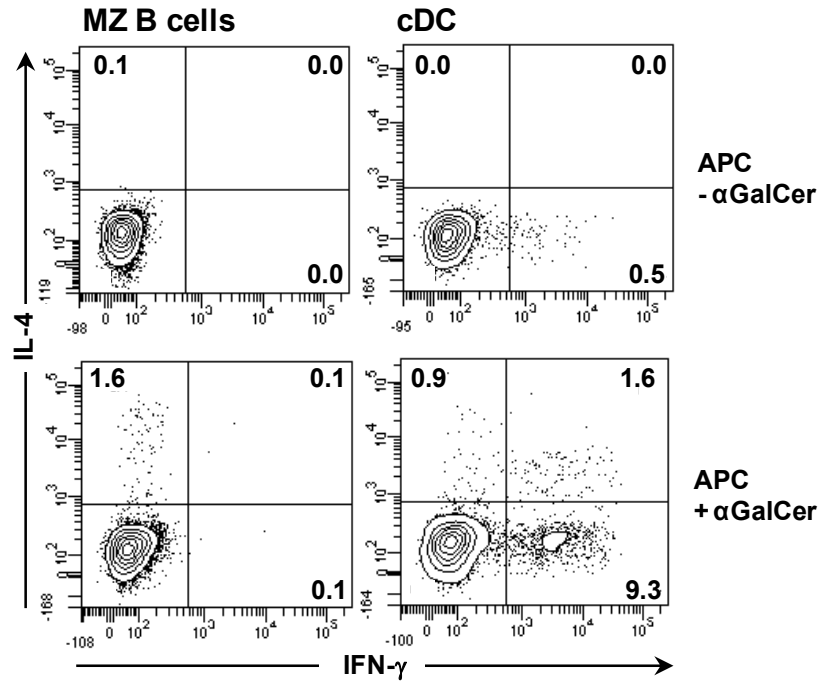


Figure 5.8 MZ B cells can efficiently present lipids to NKT cells *in vivo*. MZ B cells and cDCs were sorted from spleens of WT mice, *ex vivo* loaded with αGalCer (2µg/ml), washed and i.v. injected into CD1d$^{-/-}$ recipients. Before NKT cells were injected into recipient mice. After 16h spleens of CD1d$^{-/-}$ mice were removed, splenocytes were isolated and incubated for 4h with Brefeldin A. After stimulation cells were intracellularly stained for IL-4 and IFN-γ. Data are representative of two independent experiments.

CHAPTER VI

DISCUSSION

DISCUSSION

The overall goal of this thesis was to investigate the interaction of antigen presenting cells (APCs) with other cells of the immune system since on the one hand, APCs shape the immune response and on the other hand immunocytes shape the APCs.

To address this question, first the influence of IFN-β and its signaling on cell mediated immunity was investigated. I could show, that constitutive production of type I IFNs is crucial for proper antigen presentation of self antigens in steady state.

I could also demonstrate how lymphocytes, in particular B cells can shape the character and function of cDCs. When splenic cDCs have to differentiate under lymphopenic conditions, their character and their ability to present soluble antigen is drastically changed. Interestingly, already reconstitution of lymphopenic mice with soluble immunoglobulins was able to rescue the ability to present soluble antigen despite the absence of mature T and B lymphocytes.

Special anatomical location and access to antigen may also influence character of immune response. Therefore in this context two types of APCs - cDCs and MZ B cells were compared. Both are very potent stimulators of NKT cells expansion. Nevertheless, the type of immune response they induce is clearly distinct. MZ B cells are responsible for early IL-4 production by NKT cells, while cDCs trigger abundant IFN-γ production. This observation may critically contribute to the explanation of the paradigm of T_H1/T_H2 regulation.

6.1 Influence of constitutive production of type I IFNs on antigen presentation ability of splenic cDCs

IFNs are found in high amounts in cells exposed to viruses. They were first characterized and named as such on the basis of their antiviral activity. It is now well known that IFNs have widely overlapping, pleiotropic and immunomodulatory effects and their production is not the sole preserve of viral infections but they are also induced in response to bacterial and parasitic infections (16, 19, 158). IFNs represent important immunomodulators for the innate as well as the adaptive arm of the immune system (19, 21). They exert broad regulatory effects and various subtypes of DCs are affected by these cytokines (16, 19-21, 158). For instance, IFN-α can promote antigen cross-presentation by enhancing endosomal processing, up-regulating the expression of co-stimulatory molecules and augmenting dendritic cell viability in settings of viral

infection (21, 23, 24, 131, 159, 160). In addition, direct stimulation of T cells by IFN-α has been shown to be essential for efficient induction of cross-priming (24). Furthermore, IFN-α/β were described as crucial survival factors for activated T cells (26). Importantly apart from that, even in the absence of infection spontaneous low level production of IFN-β has been shown to occur (16).

The host response elicited by IFNs is largely dependent on signal strength. Most of the studies carried out to date have focused on the cellular effects induced by the high levels of IFNs elicited under inflammatory conditions. However, whether the low levels of IFNs produced under non-inflammatory conditions have an important housekeeping immune function is not known. This work shows now that the low but constitutive production of IFN-β is necessary for maintaining DCs in a state competent for antigen presentation. Compared to those from WT mice, DCs freshly isolated from spleens of IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were found to be highly impaired in antigen presentation to $CD4^+$ and $CD8^+$ T cells. This defect could in part be rectified with exogenous rIFN-β or through *in vivo* induction of IFN-α using synthetic dsRNA. Interestingly, restoration of function was possible with extremely low amounts of rIFN-β, probably mimicking amounts produced constitutively. Failure of IFN-$\beta^{-/-}$ DCs cultured with high rIFN-β levels to activate T cell proliferation might be attributed to negative feedback mechanisms activated by a strong IFN-β signal.

The function of DCs is not only influenced by cytokines present in their environment, but also by other cells of the immune system, particularly T cells. Therefore, splenic cDCs isolated from IFN-β deficient mice theoretically could be altered due to an effect of IFN-β deficiency on T cells. It was shown before that IFN-α and IFN-β have direct effect on activated T cells and prevent their death during inflammatory conditions (26). Activated T cells can provide feedback signals to DCs and induce their maturation. This can be mediated by cytokines produced by T cells as well as by cell-cell interactions, including CD40L-CD40 interactions (161). However, this phenomenon is unlikely to play a significant role in steady state conditions because of the lack of activated T cells. T cell mediated conditioning of DCs *via* B7-H1 in homeostasis has recently been shown to play an important role in inducing DC maturation (162). However, as surface expression of B7-H1 was not altered on IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cDCs in comparison to WT cDCs, one may assume that this is not the case in situation here. Moreover, I demonstrated that rIFN-β treatment *in vitro* can regulate cDCs function and Hsp70 levels.

Potential roles of IFNs in antigen presentation have previously been postulated. However, as mentioned above such effects have only been observed for high levels of IFNs reminiscent of inflammation. For instance, induction of IFNs in DCs by dsRNA and LPS *in vitro* was shown to up-regulate co-stimulatory and MHC molecules hence enhancing their ability to activate $CD8^+$ T cells (20). Conversely, results presented here show that the antigen presentation defects in splenic IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ DCs are neither due to low expression of co-stimulatory and MHC molecules nor a block in antigen capture and processing. I demonstrate that the defect is due to a blockade in a step downstream of antigen processing: MHC/peptide complex formation. Furthermore, the data presented here strongly suggest that impairment in the MHC/peptide complex formation step caused by the absence of IFNs signaling is due to down-regulation of Hsp70.

The 70kD Hsps Hsp70.1 and Hsp70.3 belong to a larger, highly homologous and conserved gene family whose expression can be significantly induced in response to a number of pathophysiological conditions including pathogen exposure (163). In addition to a generalized role in protein folding and transport, the proteins also have distinct functions in the promotion of antigen processing and presentation (105-108). Hsp70 proteins are involved in chaperoning proteins/peptides during degradation and during antigen presentation *via* MHC I as well as *via* MHC II. Hsp70 has been shown to physically associate with the transporter associated with antigen processing (TAP) hence enabling efficient loading of chaperoned peptides onto MHC I molecules (105, 106, 109). In addition, a role for Hsp70 in antigen presentation by MHC II molecules has also been described (105, 111).

By microarray and qRT-PCR analysis, I found that in the absence of a functional IFNs system *in vivo*, the expression of Hsp70.1 and Hsp.70.3 in splenic cDCs was significantly down-regulated. In addition, partial blockade of Hsp70 protein in WT cDCs using the pharmacological inhibitor DSG resulted in a diminished ability to activate T cells. This correlated with a decrease in the surface MHC/peptide complexes on WT splenic DCs after treatment with DSG. These results were further substantiated when I analyzed the ability of cDCs from Hsp70.1/3$^{-/-}$ mice to present soluble OVA to naive $CD4^+$ and $CD8^+$ T cells. The function of such cDCs was highly impaired when compared to WT cDCs, suggesting that indeed altered levels of Hsp70 in IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cDCs are responsible for impaired antigen presentation in the steady state.

From the data presented here, one could expect that in the absence of chaperones, like Hsp70, when formation of antigenic MHC/peptide complexes is impaired, total levels of surface MHC molecules should be diminished. Nevertheless, experiments show no differences between WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cDCs in total surface MHC I and MHC II expression. Likewise, the analysis of the surface phenotype of Hsp70.1/3$^{-/-}$ cDCs showed no significant difference in comparison to WT cDCs (data not shown). Possibly, Hsp70 might be required for efficient presentation of particular peptides, like the ones employed in our study, but not for the others. Therefore, lack of Hsp70 might not be reflected by changes of total surface MHC levels. However, the exact molecular reason for this phenomenon remains to be elucidated.

The discovery that basal IFNs production regulates efficiency of MHC/peptide complex formation *via* the expression of Hsp70 is a novel and important finding not only in the context of pathogen recognition but also in homeostasis. Not all pathogens are associated with robust IFNs production. Therefore by sustaining Hsp70 expression, constitutively produced IFNs probably ensure that DCs are kept in a primed state for efficient presentation of antigens from such pathogens. An equally or more important function could be in the maintenance of tolerance to self antigens. DCs that capture and present antigen under non-inflammatory conditions are generally believed to acquire tolerogenic properties and generate regulatory T lymphocytes that potentiate tolerogenic responses.

6.2 Cellular composition of the spleen imprints the character and function of cDCs

The immune system is a highly dynamic network, where specialized cells interact with each other to elicit either protective immune responses or maintain tolerogenic conditions. These interactions include cell-cell interactions but also interactions of soluble ligands with their cellular receptors, which additionally may be influenced by particular anatomical locations. For instance, as I have shown above, the development and function of cDCs is critically controlled by soluble molecules, like IFN-β. In the second part of my thesis, I addressed the question how the cellular composition of the spleen, especially presence of B cells and their products imprints the character and antigen presenting capacities of splenic cDCs.

Impaired function of splenic cDCs from RAG$^{-/-}$ mice to present soluble antigen was already shown before (143), although the authors worked with skin-derived DCs and

tested their antigen presenting ability in different experimental system. In this work, the authors showed that reconstitution of RAG deficient mice with T cells could rescue the function of DCs. Thus, they hypothesized that DCs need T cells for functional maturation *in vivo* (143).

In my work, I could extend the functional deficiency described above to cDCs found in the spleen of RAG deficient mice. I decided to go deeper into the molecular details and possibly find independent mechanistic explanations for the impaired function of such DCs.

First, the expression of various surface molecules did not reveal any significant differences between $RAG^{-/-}$ and WT cDCs, except that reproducibly I could detect higher levels of MHC I and MHC II molecules on cDCs from RAG deficient mice in comparison to WT cDCs. A first possibility, namely that $RAG^{-/-}$cDCs were more mature than WT cDCs, could be excluded on the basis of comparable phagocytic activity and similar levels of co-stimulatory molecules. In addition, cDCs that would have matured due to inflammatory stimuli would up-regulate the expression of MHC I and MHC II molecules to a much greater extend than found here. Finally, the analysis of the transcriptional profile did not reveal a significant difference in maturation between WT and $RAG^{-/-}$ splenic cDCs.

However, from the transcriptional profiling of both types of cDCs new insights into the nature of the $RAG^{-/-}$ cDCs deficiency could be gained. Three groups of genes were differentially expressed in splenic cDCs in the absence of T and B cells.

One group of genes was encoding endo-lysosmal enzymes, which are responsible for degradation of antigen. Their up-regulation in $RAG^{-/-}$ cDCs strongly suggested a more aggressive degradation than in WT cDCs. Indeed, this hypothesis was confirmed experimentally by flow cytometry employing DQ-OVA or imunoblot with biotinylated OVA. Consistent with this idea, efficient cross-priming of $CD8^{+}$ T cells was shown to require the presence of antigen storage compartments in DCs (93). Such compartments were described as lysosomal-like organelles. Therefore highly aggressive degradation of antigen in $RAG^{-/-}$ cDCs would lead to a reduction of this intracellular antigen depot, and thus a reduction in cross-priming efficiency.

In the second group of genes C1q complement family members were found. Beyond their very well established role in activation of the classical complement pathway, C1q components are also responsible for clearance of apoptotic debris (155). Therefore, it was possible that $RAG^{-/-}$ cDCs are specialized to presentat of particular antigens, like

cell-associated antigens derived from apoptotic cells. Indeed, presentation of antigen associated with apoptotic bodies was equally efficient by cDCs from both types of mice.

Interestingly, when I tested the spleen of RAG deficient mice for the number of apoptotic cells, I could detect much more of them than in WT spleen. Thus, the lack of particular cells in the spleen of RAG deficient mice lead to an increase in the numbers of apoptotic cells, possibly due to a lack of survival signals derived from T or B cells. This might explain, why the percentage of cDCs in the spleen of such mice is similar to WT mice. The total number of cDCs needs to be dramatically reduced since the cellularity of $RAG^{-/-}$ spleen is also strongly reduced. As a consequence, $RAG^{-/-}$ cDCs may favor to phagocytose dead cells and present cell associated antigens.

The third group of genes concerned cell surface receptors responsible for up-take of soluble antigen (like the mannose receptor) as well as Fc receptors for immunoglobulins. Indeed, the ability of $RAG^{-/-}$ mice to acquire soluble OVA, was slightly enhanced in comparison to WT cDCs. On the other hand, high expression of Fc receptors could indicate that signaling *via* these receptors might be important for the functional fine-tuning of splenic cDCs. Engagement of FcγRIIB on DCs was shown to enhance their maturation as well as their ability to present antigen *via* both MHC I and MHC II (147, 148).

Fc receptors are normally expressed as receptor pairs - activating together with inhibiting - and in steady state there is maintenance of a strict balance between those two forces. Thus, disturbances of this homeostatsis may lead to improper activation/inhibition of cellular functions. The absence of B cells or immunoglobulins produced by them, might therefore explain the distorted function of splenic cDCs from $RAG^{-/-}$ mice.

Indeed, reconstitution of $RAG^{-/-}$ mice with WT B cells rescued the impaired ability of splenic cDCs to cross-present soluble OVA. Moreover when purified immunoglobulins were administered, the same effect could be observed. The latter finding excluded the possibility that the rescue of cDCs function by B cells was due to production of cytokines or other mediators by such cells.

Therefore, the most likely explanation of the impaired APC function of cDCs from $RAG^{-/-}$ mice is the absence of immunoglubilins in the circulation. The binding of immunoglobulins to the FC receptors in WT mice could either positively drive the cDCs into a maturation state, that allows efficient presentation of soluble antigens.

However, I think it is more likely that such immunoglobulins exert an inhibitory signal, e.g. by binding to FcγRII that tames the aggressive degradation mechanisms in WT cDCs. This hypothesis is testable. It would predict that splenic cDCs from recombinant mice that lack the required activating or inhibitory Fc receptors will show the same phenotype as splenic cDCs from $RAG^{-/-}$ mice.

An aspect that has been ignored so far is the concurrent influence of T cells that has been shown for skin-derived DCs (143). For the time being, it cannot be excluded that reconstitution of $RAG^{-/-}$ mice with T cells would similarly recover impaired cross-presentation by $RAG^{-/-}$ splenic cDCs. Such experiments are crucial for a complete picture. Possibly, the presence of both, B and T cells is essential for maintenance of proper function of splenic cDCs. Yet, this still remains to be elucidated.

Together, I could demonstrate that the absence of T and B lymphocytes changes the character and function of cDCs in the spleen. The deviant character of cDCs from RAG deficient mice could exemplify the adaptation to particular cellular composition and particular molecules present in the spleen. Therefore, one should be aware of such limitations when using of $RAG^{-/-}$ mice for experiments of T cell regulation, tolerance induction and APC - T cell interaction in general. On the other hand it represents the most beautiful example of the complex homeostatic interaction and dependency of the innate and adaptive immune system.

6.3 MZ B cells efficiently stimulate NKT cells proliferation and production of IL-4

Expression of the MHC I b molecule CD1d by MZ B cells had already been described before (64, 150-152, 164, 165). The exceedingly high expression of CD1d was revealed by the direct comparison of various cell types in the present work. Since CD1d is the restricting molecule for NKT cells, the capacity of MZ B cells to stimulate NKT cells was tested. Interestingly, MZ B cell sensitized with αGalCer were very efficient in priming NKT cells *in vitro*. In fact, they were better than cDCs that also expressed considerable amounts of CD1d. The two other splenic B cell populations - B1 and B2 cells - completely failed to activate NKT cells although they expressed CD1d to some extend.

A recent study (151) showed that MZ B cells require DCs to activate NKT cells, although this work had some flaws since the purity of NKT cells employed was only

around 50%. However, this raises the question whether my results are also due to DCs present in the cell preparations. I think, I can rule out this possibility. NKT cells were sorted to purity greater than 98% as were the APCs. In addition, one would expect that the other B cell populations would also have triggered NKT cells under these conditions. This was clearly not the case. Moreover, in this work the authors did not observe T_H1/T_H2 dichotomy when different APCs were used for NKT cells stimulation. Since in my case this is a very robust result, the controversy is most likely due to undefined cell populations in the particular assays employed.

In my hands, co-culture of αGalCer sensitized cDCs with NKT cells reproducibly induced the production of IFN-γ in many of the responder cells while IL-4 producing cells were scarce under these conditions. In contrast, MZ B cells induced mainly IL-4, especially early after stimulation. In addition, MZ B cells failed to induce secretion of IFN-γ by NKT cells. The capacity to induce proliferation of NKT cells was comparable for both, cDCs and MZ B cells. The differentiation of NKT cells was drastically influenced by the type APC employed. MZ B cells directed the response into a direction that should favor T_H2 response while cDCs induced T_H1 like conditions.

In similar published studies, bulk sorted B cells were employed to test their stimulatory capacity for NKT cells (164). Such B cells alone were able to activate NKT cells. However, stimulatory capacity was 2- to 3- fold lower than that of cDCs. Interestingly, the authors observed a dichotomy of T_H1 and T_H2 differentiation dependent of whether the NKT cells were stimulated by DCs or B cells. Similarly Kitamura et al.(166) showed that αGalCer induces early B-cell activation through production of IL-4 by NKT cells. Again the authors tested a bulk $B220^+$ B cell population only.

Such findings were also reminiscent of the work by Stockinger et al. (167). They could observe a difference in the cytokine pattern elicited in $CD4^+$ T cells dependent of whether they used DCs or B cells as APC.

All such findings lack a confirmation of relevance *in vivo*. In contrast, I provide an *in vivo* confirmation of my findings. Injection of sensitized cDCs or MZ B cells into CD1d deficient mice that had been reconstituted with NKT cells or into WT mice (data not shown) revealed that the dichotomy observed *in vitro* was even more pronounced *in vivo*. MZ B cells exclusively stimulated NKT cells to differentiate into IL-4 producers, while cDCs almost exclusively induced IFN-γ production in NKT

cells. This indicates that the reaction I have observed here might have important implications for the initiation and regulation of defensive immune responses *in vivo*.

The direction of the NKT cells response clearly depended on the type of APC that was presenting an identical antigen *via* the same CD1d molecule. Hence, I hypothesized that such APCs should be distinct in the co-stimulatory molecules that are engaging the NKT cells. Thus, several surface receptors and ligands on APCs as well as on NKT cells were examined. Interestingly, differential expression of two ligands, GITRL and ICOSL, on MZ B cells in comparison to cDCs was observed. Both of them were significantly higher expressed on MZ B cells.

The role of GITR in NKT cells activation is still controversial. Some studies showed that engagement of GITR leads to co-stimulation of NKT cells and increases production of IFN-γ and IL-4 (156). In contrast, others demonstrate that GITR engagement elicits co-inhibitory activity for antigen-induced NKT cells (168). This controversy appears not to be entirely resolved yet.

In my study, blockage of GITR by antibodies did not alter the ability of NKT cells to proliferate in response to both, MZ B cells and cDCs. However, IFN-γ production after stimulation of NKT cells by cDCs was significantly inhibited while the response of IL-4 elicited by MZ B cells was not influenced. Therefore, the GITR/GITRL pathway appears to be dispensable during MZ B cells induced NKT cells differentiation. In contrast, for differentiation of NKT cells into T_H1 supporting cells this interaction appears to be essential.

ICOS/ICOSL interaction was shown to have a major role in the expansion of T_H2 immune responses (155, 157). *In vivo* blockade of ICOS affects T_H2 effector functions (157, 169). Presence of a functional ICOS/ICOSL pathway was shown to be crucial for the development of allergic airway disease and to development of allergen specific T_H2 cells (155, 157). ICOS is known to be highly expressed on T cells after their initial activation (170), therefore ICOS/ICOSL interaction might be most influential early during MZ B cells and NKT cells interactions. Moreover characterization of $ICOS^{-/-}$ mice revealed that T cells isolated from these animals are not able to produce IL-4 (171).

Importantly, I showed that inhibition of ICOSL on MZ B cells leads to complete abrogation of NKT cells proliferation and IL-4 production. This experiment strongly suggested that MZ B cells *via* ICOS/ICOSL interaction drive NKT cells proliferation and early IL-4 production that might be decisive for elicitation a T_H2 type of response.

The scenario that starts to appear based on the present results is as follows: MZ B cells act as professional antigen presenting cells of the marginal zone. Moreover, the MZ B cells play a special role with regards to regulation of immune responses. NKT cell stimulated by MZ B cells might induce a cytokine milieu that drives naive $CD4^+$ T helper cells to differentiate into T_H2 cells. Presence of functional ICOS/ICOSL pathway is necessary for both NKT cells expansion as well as production of IL-4 by NKT cells.

Thus, lipids of particular blood borne pathogens that have a high preference to interact with MZ B cells will lead to a T_H2 inducing milieu by the NKT cells. In contrast, lipids of most blood borne pathogens will interact with cDCs in the marginal zone and thus induce a T_H1 milieu. In consequence, strong triggering of NKT towards one or the other direction by either MZ B cells or cDCs will have a strong influence on the subsequent immune reaction. Hence, this interaction should be decisive of whether the response will be protective or detrimental.

6.4 Concluding remarks

My work reflected on the importance of APCs for the immune system. First, I could demonstrate the importance of IFN-β for the development of cDCs *via* up-regulation of Hsp70.1 and Hsp70.3 under steady state conditions. The identification of such heat shock proteins as interferon regulated genes had been missed thus far. Up-regulation is only observed when low amounts of IFN-β are applied as they are found under steady state. Amounts of IFN-β, that are encountered under inflammatory conditions, led to a negative feedback regulation of Hsp70.1/3.

Second, immunoglobulins appear to be important regulatory molecules for the maturation of cDCs. Antibodies were clearly believed to be involved in antigen presentation because antibody/antigen complexes might be preferentially endocytosed *via* Fc receptors and engagement of these receptors might provide activating signals. A direct regulatory function of not complexed immunoglobulins on the differentiation of cDCs is novel and unexpected.

Finally, I could provide suggestive evidence that MZ B cells are involved in the decision for the T_H1/T_H2 bias. I could show that such B cells trigger NKT cells to proliferate and to differentiate into IL-4 producers. Since NKT cells are known that

they respond extremely rapidly upon antigen stimulation, this interaction might be a key reaction for the regulation of protective immune responses.

Thus, I believe that my thesis is an important corner stone in the understanding of the complex regulatory mechanisms shaping the immune system and its reactions.

CHAPTER VII

SUMMARY

SUMMARY

Antigen presenting cells (APCs) represent one of the most important cell types of the immune system. They are crucial for the initiation of a protective adaptive immune responses as well as for maintenance of tolerogenic conditions and tissue homeostasis. Therefore, how the function of APCs is regulated represents an extremely important aspect in understanding the decisions made during an immune response. The work I presented here, addressed the questions how single molecules, cellular composition of the spleen and particular anatomical location influence the character and outcome of an immune response. It was of particular interest how the function of splenic APCs can be established in steady state conditions.

Taken together the results presented here show that:

- IFN-β and type I IFNs signaling are essential factors for proper antigen presentation by splenic cDCs in steady state;
- Absence of IFN-β can be recovered by already very low amounts of exogenous IFN-β. This is reminiscent of low amounts of IFN-β constitutively produced under steady state conditions;
- Lack of IFN-β or type I IFNs signaling leads to down-regulation of members of Hsp70 family in splenic cDCs, which serve as molecular chaperones during antigen presentation *via* both MHC I and MHC II;
- The antigen presenting ability of Hsp70.1/3$^{-/-}$ cDCs revealed the same phenotype as IFN-β$^{-/-}$ and IFNAR$^{-/-}$cDCs. This confirmed that constitutive levels of type I IFNs by regulating the Hsp70 levels, ensures that antigen presentation in steady state works properly and efficiently;
- These data suggest that constitutively produced low amounts of type I IFNs are important not only for presentation of pathogen derived antigens, which do not induce robust IFNs response, but also for presentation of self antigens in steady state, which might be crucial for maintenance of tolerance and tissue homeostasis;

- Absence of T and B cells has important implications on character and function of splenic cDCs. They are impaired in their presentation capacity for soluble antigen;
- cDCs from $RAG^{-/-}$ mice exhibit a more aggressive antigen degradation pathway than cDCs from WT mice;
- Reconstitution of $RAG^{-/-}$ mice with soluble immunoglobulins is sufficient to recover impaired function of cDCs to present soluble antigen;
- Particular anatomical location and high expression of CD1d on the surface of MZ B cells enable them to be very efficient activators of NKT cells *in vitro* and *in vivo*;
- Compared to cDCs, stimulation of NKT cells by MZ B cells lead to exclusive production of IL-4;
- To stimulate NKT cell proliferation and IL-4 production MZ B cells utilize the ICOS/ICOSL co-stimulation pathway;
- Different anatomical location and access to antigen seems to be decisive for the nature of induced immune response, cDCs rather favor T_H1 conditions, whereas MZ B cells T_H2;

These results shed a new light on how the antigen presentation by splenic cDCs is regulated in homeostatic conditions. Importantly, they revealed the sensitivity of this process on a single soluble molecule, i.e. IFN-β. Studying and characterizing of cDCs from $RAG^{-/-}$ mice revealed an unexpected critical role of B cells and immunoglobulins and their influence on the function of cDCs. Furthermore, results presented here may contribute to a deeper understanding of the initiation of the T_H1 versus T_H2 bias. This is decisive for the type of induced immune response, T_H1 favor cell mediated immunity and T_H2 will promote humoral responses. Thus, my work underscores the complexity of the cellular and molecular requirements for the development of a functional immune system. But I was also able to provide new mechanistic insights into such regulatory mechanisms.

REFERENCES

REFERENCE LIST

1. Paul, W.E. 2008. Fundamental Immunology, Sixth edition. Wolters Kluwer, Lippincott Williams&Wilkins publishers.

2. Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature* 449:819-826.

3. Vivier, E. and B. Malissen. 2005. Innate and adaptive immunity: specificities and signaling hierarchies revisited. *Nat Immunol* 6:17-21.

4. Martinon, F., A. Mayor, and J. Tschopp. 2009. The inflammasomes: guardians of the body. *Annu.Rev Immunol* 27:229-265.

5. Janeway, C. A., Jr. and R. Medzhitov. 2002. Innate immune recognition. *Annu.Rev Immunol* 20:197-216.

6. Mogensen, T. H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin.Microbiol Rev* 22:240-73, Table.

7. Sjoberg, A. P., L. A. Trouw, and A. M. Blom. 2009. Complement activation and inhibition: a delicate balance. *Trends Immunol* 30:83-90.

8. Carroll, M. C. 2004. The complement system in regulation of adaptive immunity. *Nat Immunol* 5:981-986.

9. Carroll, M. C. 1998. The role of complement and complement receptors in induction and regulation of immunity. *Annu.Rev Immunol* 16:545-568.

10. Carroll, M. C. and M. B. Fischer. 1997. Complement and the immune response. *Curr.Opin.Immunol* 9:64-69.

11. Isaacs, A. and J. Lindenmann. 1957. Virus interference. I. The interferon. *Proc.R.Soc.Lond B Biol Sci.* 147:258-267.

12. Smith, P. L., G. Lombardi, and G. R. Foster. 2005. Type I interferons and the innate immune response--more than just antiviral cytokines. *Mol.Immunol* 42:869-877.

13. Stetson, D. B. and R. Medzhitov. 2006. Type I interferons in host defense. *Immunity* 25:373-381.

14. Tovey, M. G., C. Lallemand, and G. Thyphronitis. 2008. Adjuvant activity of type I interferons. *Biol Chem.* 389:541-545.

15. Mogensen, K. E., M. Lewerenz, J. Reboul, G. Lutfalla, and G. Uze. 1999. The type I interferon receptor: structure, function, and evolution of a family business. *J Interferon Cytokine Res* 19:1069-1098.

16. Taniguchi, T. and A. Takaoka. 2001. A weak signal for strong responses: interferon-alpha/beta revisited. *Nat Rev Mol.Cell Biol* 2:378-386.

17. Takaoka, A., Y. Mitani, H. Suemori, M. Sato, T. Yokochi, S. Noguchi, N. Tanaka, and T. Taniguchi. 2000. Cross talk between interferon-gamma and -alpha/beta signaling components in caveolar membrane domains. *Science* 288:2357-2360.

18. Theofilopoulos, A. N., R. Baccala, B. Beutler, and D. H. Kono. 2005. Type I interferons (alpha/beta) in immunity and autoimmunity. *Annu.Rev Immunol* 23:307-336.

19. Le Bon, A. and D. F. Tough. 2002. Links between innate and adaptive immunity via type I interferon. *Current Opinion in Immunology* 14:432-436.

20. Hoebe, K., E. Janssen, and B. Beutler. 2004. The interface between innate and adaptive immunity. *Nature Immunology* 5:971-974.

21. Montoya, M., G. Schiavoni, F. Mattei, I. Gresser, F. Belardelli, P. Borrow, and D. F. Tough. 2002. Type I interferons produced by dendritic cells promote their phenotypic and functional activation. *Blood* 99:3263-3271.

22. Asselin-Paturel, C. and G. Trinchieri. 2005. Production of type I interferons: plasmacytoid dendritic cells and beyond. *J Exp.Med* 202:461-465.

23. Le Bon, A. and D. F. Tough. 2008. Type I interferon as a stimulus for cross-priming. *Cytokine Growth Factor Rev* 19:33-40.

24. Le Bon, A., V. Durand, E. Kamphuis, C. Thompson, S. Bulfone-Paus, C. Rossmann, U. Kalinke, and D. F. Tough. 2006. Direct stimulation of T cells by type IIFN enhances the CD8(+) T cell response during cross-priming. *Journal of Immunology* 176:4682-4689.

25. Le Bon, A., C. Thompson, E. Kamphuis, V. Durand, C. Rossmann, U. Kalinke, and D. F. Tough. 2006. Cutting edge: Enhancement of antibody responses through direct stimulation of B and T cells by type IIFN. *Journal of Immunology* 176:2074-2078.

26. Marrack, P., J. Kappler, and T. Mitchell. 1999. Type I interferons keep activated T cells alive. *Journal of Experimental Medicine* 189:521-529.

27. Presland, R. B. and R. J. Jurevic. 2002. Making sense of the epithelial barrier: what molecular biology and genetics tell us about the functions of oral mucosal and epidermal tissues. *J Dent.Educ.* 66:564-574.

28. Steinman, R. M. and Z. A. Cohn. 2007. Pillars Article: Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J. Exp. Med.1973. 137: 1142-1162. *J Immunol* 178:5-25.

29. Merad, M. and M. G. Manz. 2009. Dendritic cell homeostasis. *Blood* 113:3418-3427.

30. Wu, L. and A. Dakic. 2004. Development of dendritic cell system. *Cell Mol.Immunol* 1:112-118.

31. Segura, E. and J. A. Villadangos. 2009. Antigen presentation by dendritic cells in vivo. *Curr.Opin.Immunol* 21:105-110.

32. Villadangos, J. A., P. Schnorrer, and N. S. Wilson. 2005. Control of MHC class II antigen presentation in dendritic cells: a balance between creative and destructive forces. *Immunol Rev* 207:191-205.

33. Becker, Y. 2003. Milestones in the research on skin epidermal Langerhans/dendritic cells (LCs/DCs) from the discovery of Paul Langerhans 1868-1989. *Virus Genes* 26:131-134.

34. Poulin, L. F., S. Henri, B. de Bovis, E. Devilard, A. Kissenpfennig, and B. Malissen. 2007. The dermis contains langerin+ dendritic cells that develop and function independently of epidermal Langerhans cells. *J Exp.Med* 204:3119-3131.

35. Ginhoux, F., M. P. Collin, M. Bogunovic, M. Abel, M. Leboeuf, J. Helft, J. Ochando, A. Kissenpfennig, B. Malissen, M. Grisotto, H. Snoeck, G. Randolph, and M. Merad. 2007. Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J Exp.Med* 204:3133-3146.

36. Liu, K., G. D. Victora, T. A. Schwickert, P. Guermonprez, M. M. Meredith, K. Yao, F. F. Chu, G. J. Randolph, A. Y. Rudensky, and M. Nussenzweig. 2009. In vivo analysis of dendritic cell development and homeostasis. *Science* 324:392-397.

37. Dudziak, D., A. O. Kamphorst, G. F. Heidkamp, V. R. Buchholz, C. Trumpfheller, S. Yamazaki, C. Cheong, K. Liu, H. W. Lee, C. G. Park, R. M. Steinman, and M. C. Nussenzweig. 2007. Differential antigen processing by dendritic cell subsets in vivo. *Science* 315:107-111.

38. Nakano, H., M. Yanagita, and M. D. Gunn. 2001. CD11c(+)B220(+)Gr-1(+) cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp.Med* 194:1171-1178.

39. Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Briere, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* 2:1144-1150.

40. Bjorck, P. 2001. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor-treated mice. *Blood* 98:3520-3526.

41. O'Keeffe, M., H. Hochrein, D. Vremec, I. Caminschi, J. L. Miller, E. M. Anders, L. Wu, M. H. Lahoud, S. Henri, B. Scott, P. Hertzog, L. Tatarczuch, and K. Shortman. 2002. Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus. *J Exp.Med* 196:1307-1319.

42. Zhang, J., A. Raper, N. Sugita, R. Hingorani, M. Salio, M. J. Palmowski, V. Cerundolo, and P. R. Crocker. 2006. Characterization of Siglec-H as a novel endocytic receptor expressed on murine plasmacytoid dendritic cell precursors. *Blood* 107:3600-3608.

43. Wu, L. and Y. J. Liu. 2007. Development of dendritic-cell lineages. *Immunity* 26:741-750.

44. Traver, D., K. Akashi, M. Manz, M. Merad, T. Miyamoto, E. G. Engleman, and I. L. Weissman. 2000. Development of CD8alpha-positive dendritic cells from a common myeloid progenitor. *Science* 290:2152-2154.

45. Naik, S., D. Vremec, L. Wu, M. O'Keeffe, and K. Shortman. 2003. CD8alpha+ mouse spleen dendritic cells do not originate from the CD8alpha- dendritic cell subset. *Blood* 102:601-604.

46. Wu, L., A. D'Amico, H. Hochrein, M. O'Keeffe, K. Shortman, and K. Lucas. 2001. Development of thymic and splenic dendritic cell populations from different hemopoietic precursors. *Blood* 98:3376-3382.

47. Liu, K., C. Waskow, X. T. Liu, K. H. Yao, J. Hoh, and M. Nussenzweig. 2007. Origin of dendritic cells in peripheral lymphoid organs of mice. *Nature Immunology* 8:578-583.

48. Naik, S. H., P. Sathe, H. Y. Park, D. Metcalf, A. I. Proietto, A. Dakic, S. Carotta, M. O'Keeffe, M. Bahlo, A. Papenfuss, J. Y. Kwak, L. Wu, and K. Shortman. 2007. Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. *Nat Immunol* 8:1217-1226.

49. Shortman, K. and S. H. Naik. 2007. Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 7:19-30.

50. Schnorrer, P., G. M. Behrens, N. S. Wilson, J. L. Pooley, C. M. Smith, D. El Sukkari, G. Davey, F. Kupresanin, M. Li, E. Maraskovsky, G. T. Belz, F. R. Carbone, K. Shortman, W. R. Heath, and J. A. Villadangos. 2006. The dominant role of CD8+ dendritic cells in cross-presentation is not dictated by antigen capture. *Proc.Natl Acad.Sci.U.S A* 103:10729-10734.

51. Villadangos, J. A. and P. Schnorrer. 2007. Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo. *Nat Rev Immunol* 7:543-555.

52. Young, L. J., N. S. Wilson, P. Schnorrer, A. Proietto, T. ten Broeke, Y. Matsuki, A. M. Mount, G. T. Belz, M. O'Keeffe, M. Ohmura-Hoshino, S. Ishido, W. Stoorvogel, W. R. Heath, K. Shortman, and J. A. Villadangos. 2008. Differential MHC class II synthesis and ubiquitination confers distinct antigen-presenting properties on conventional and plasmacytoid dendritic cells. *Nat Immunol* 9:1244-1252.

53. Villadangos, J. A. and L. Young. 2008. Antigen-presentation properties of plasmacytoid dendritic cells. *Immunity* 29:352-361.

54. Kawai, T. and S. Akira. 2009. The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int.Immunol* 21:317-337.

55. Takahashi, K., W. E. Ip, I. C. Michelow, and R. A. Ezekowitz. 2006. The mannose-binding lectin: a prototypic pattern recognition molecule. *Curr.Opin.Immunol* 18:16-23.

56. Geijtenbeek, T. B., S. J. van Vliet, A. Engering, B. A. 't Hart, and Y. van Kooyk. 2004. Self- and nonself-recognition by C-type lectins on dendritic cells. *Annu.Rev Immunol* 22:33-54.

57. Kawai, T. and S. Akira. 2005. Toll-like receptor downstream signaling. *Arthritis Res Ther.* 7:12-19.

58. Ashour, H. M. and T. M. Seif. 2007. The role of B cells in the induction of peripheral T cell tolerance. *J Leukoc Biol* 82:1033-1039.

59. Raimondi, G., I. Zanoni, S. Citterio, P. Ricciardi-Castagnoli, and F. Granucci. 2006. Induction of peripheral T cell tolerance by antigen-presenting B cells. I. Relevance of antigen presentation persistence. *J Immunol* 176:4012-4020.

60. Hardy, R. R. and K. Hayakawa. 2001. B cell development pathways. *Annu.Rev Immunol* 19:595-621.

61. Martin, F. and J. F. Kearney. 2000. B-cell subsets and the mature preimmune repertoire. Marginal zone and B1 B cells as part of a "natural immune memory". *Immunol Rev* 175:70-79.

62. Lopes-Carvalho, T. and J. F. Kearney. 2004. Development and selection of marginal zone B cells. *Immunol Rev* 197:192-205.

63. Martin, F. and J. F. Kearney. 2002. Marginal-zone B cells. *Nat Rev Immunol* 2:323-335.

64. Lopes-Carvalho, T., J. Foote, and J. F. Kearney. 2005. Marginal zone B cells in lymphocyte activation and regulation. *Curr.Opin.Immunol* 17:244-250.

65. Kraal, G. 1992. Cells in the marginal zone of the spleen. *Int.Rev Cytol.* 132:31-74.

66. Cinamon, G., M. A. Zachariah, O. M. Lam, F. W. Foss, Jr., and J. G. Cyster. 2008. Follicular shuttling of marginal zone B cells facilitates antigen transport. *Nat Immunol* 9:54-62.

67. Woodland, D. L. and R. W. Dutton. 2003. Heterogeneity of CD4(+) and CD8(+) T cells. *Curr.Opin.Immunol* 15:336-342.

68. Castellino, F. and R. N. Germain. 2006. Cooperation between CD4+ and CD8+ T cells: when, where, and how. *Annu.Rev Immunol* 24:519-540.

69. Steinman, L. 2007. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* 13:139-145.

70. Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo. 2009. IL-17 and Th17 Cells. *Annu.Rev Immunol* 27:485-517.

71. Shevach, E. M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity* 30:636-645.

72. Bendelac, A., P. B. Savage, and L. Teyton. 2007. The biology of NKT cells. *Annu.Rev Immunol* 25:297-336.

73. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu.Rev Immunol* 23:877-900.

74. Macdonald, H. R. 2007. NKT cells: In the beginning... *Eur.J Immunol* 37 Suppl 1:S111-S115.

75. Taniguchi, M., M. Harada, S. Kojo, T. Nakayama, and H. Wakao. 2003. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu.Rev Immunol* 21:483-513.

76. Medzhitov, R. and C. A. Janeway, Jr. 2000. How does the immune system distinguish self from nonself?. *Semin.Immunol* 12:185-188.

77. Vyas, J. M., A. G. Van der Veen, and H. L. Ploegh. 2008. The known unknowns of antigen processing and presentation. *Nat Rev Immunol* 8:607-618.

78. Bjorkman, P. J., M. A. Saper, B. Samraoui, W. S. Bennett, J. L. Strominger, and D. C. Wiley. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329:506-512.

79. Arosa, F. A., S. G. Santos, and S. J. Powis. 2007. Open conformers: the hidden face of MHC-I molecules. *Trends Immunol* 28:115-123.

80. Shastri, N., S. Cardinaud, S. R. Schwab, T. Serwold, and J. Kunisawa. 2005. All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. *Immunol Rev* 207:31-41.

81. Cresswell, P., N. Bangia, T. Dick, and G. Diedrich. 1999. The nature of the MHC class I peptide loading complex. *Immunol Rev* 172:21-28.

82. Hammer, G. E., F. Gonzalez, E. James, H. Nolla, and N. Shastri. 2007. In the absence of aminopeptidase ERAAP, MHC class I molecules present many unstable and highly immunogenic peptides. *Nature Immunology* 8:101-108.

83. Jensen, P. E. 2007. Recent advances in antigen processing and presentation. *Nat Immunol* 8:1041-1048.

84. Strehl, B., U. Seifert, E. Kruger, S. Heink, U. Kuckelkorn, and P. M. Kloetzel. 2005. Interferon-gamma, the functional plasticity of the ubiquitin-proteasome system, and MHC class I antigen processing. *Immunol Rev* 207:19-30.

85. Kloetzel, P. M. 2004. Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII. *Nat Immunol* 5:661-669.

86. Rock, K. L., I. A. York, and A. L. Goldberg. 2004. Post-proteasomal antigen processing for major histocompatibility complex class I presentation. *Nat Immunol* 5:670-677.

87. Trombetta, E. S. and I. Mellman. 2005. Cell biology of antigen processing in vitro and in vivo. *Annu.Rev Immunol* 23:975-1028.

88. Cresswell, P., A. L. Ackerman, A. Giodini, D. R. Peaper, and P. A. Wearsch. 2005. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol Rev* 207:145-157.

89. Groothuis, T. A., A. C. Griekspoor, J. J. Neijssen, C. A. Herberts, and J. J. Neefjes. 2005. MHC class I alleles and their exploration of the antigen-processing machinery. *Immunol Rev* 207:60-76.

90. Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp.Med* 143:1283-1288.

91. Burgdorf, S., C. Scholz, A. Kautz, R. Tampe, and C. Kurts. 2008. Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. *Nat Immunol* 9:558-566.

92. Lin, M. L., Y. Zhan, J. A. Villadangos, and A. M. Lew. 2008. The cell biology of cross-presentation and the role of dendritic cell subsets. *Immunol Cell Biol* 86:353-362.

93. van Montfoort, N., M. G. Camps, S. Khan, D. V. Filippov, J. J. Weterings, J. M. Griffith, H. J. Geuze, T. van Hall, J. S. Verbeek, C. J. Melief, and F. Ossendorp. 2009. Antigen storage compartments in mature dendritic cells facilitate prolonged cytotoxic T lymphocyte cross-priming capacity. *Proc.Natl Acad.Sci.U.S A* 106:6730-6735.

94. Brown, J. H., T. S. Jardetzky, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature* 364:33-39.

95. Faure-Andre, G., P. Vargas, M. I. Yuseff, M. Heuze, J. Diaz, D. Lankar, V. Steri, J. Manry, S. Hugues, F. Vascotto, J. Boulanger, G. Raposo, M. R. Bono, M. Rosemblatt, M. Piel, and A. M. Lennon-Dumenil. 2008. Regulation of dendritic cell migration by CD74, the MHC class II-associated invariant chain. *Science* 322:1705-1710.

96. Watts, C. 2004. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. *Nat Immunol* 5:685-692.

97. Barral, D. C. and M. B. Brenner. 2007. CD1 antigen presentation: how it works. *Nat Rev Immunol* 7:929-941.

98. Bricard, G. and S. A. Porcelli. 2007. Antigen presentation by CD1 molecules and the generation of lipid-specific T cell immunity. *Cell Mol.Life Sci.* 64:1824-1840.

99. Speak, A. O., V. Cerundolo, and F. M. Platt. 2008. CD1d presentation of glycolipids. *Immunol Cell Biol* 86:588-597.

100. Stronge, V. S., M. Salio, E. Y. Jones, and V. Cerundolo. 2007. A closer look at CD1d molecules: new horizons in studying NKT cells. *Trends Immunol* 28:455-462.

101. Koch, M., V. S. Stronge, D. Shepherd, S. D. Gadola, B. Mathew, G. Ritter, A. R. Fersht, G. S. Besra, R. R. Schmidt, E. Y. Jones, and V. Cerundolo. 2005. The crystal structure of human CD1d with and without alpha-galactosylceramide. *Nat Immunol* 6:819-826.

102. Tissieres, A., H. K. Mitchell, and U. M. Tracy. 1974. Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. *J Mol.Biol* 84:389-398.

103. Pockley, A. G. 2003. Heat shock proteins as regulators of the immune response. *Lancet* 362:469-476.

104. Daugaard, M., M. Rohde, and M. Jaattela. 2007. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS Lett.* 581:3702-3710.

105. Li, Z., A. Menoret, and P. Srivastava. 2002. Roles of heat-shock proteins in antigen presentation and cross-presentation. *Curr.Opin.Immunol* 14:45-51.

106. Binder, R. J. and P. K. Srivastava. 2005. Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells. *Nat Immunol* 6:593-599.

107. Kuppner, M. C., R. Gastpar, S. Gelwer, E. Nossner, O. Ochmann, A. Scharner, and R. D. Issels. 2001. The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. *Eur.J Immunol* 31:1602-1609.

108. Manara, G. C., P. Sansoni, L. Badiali-De Giorgi, G. Gallinella, C. Ferrari, V. Brianti, F. F. Fagnoni, C. L. Ruegg, G. De Panfilis, and G. Pasquinelli. 1993. New insights suggesting a possible role of a heat shock protein 70-kD family-related protein in antigen processing/presentation phenomenon in humans. *Blood* 82:2865-2871.

109. Chen, D. and M. J. Androlewicz. 2001. Heat shock protein 70 moderately enhances peptide binding and transport by the transporter associated with antigen processing. *Immunol Lett.* 75:143-148.

110. Flechtner, J. B., K. P. Cohane, S. Mehta, P. Slusarewicz, A. K. Leonard, B. H. Barber, D. L. Levey, and S. Andjelic. 2006. High-affinity interactions between peptides and heat shock protein 70 augment CD8+ T lymphocyte immune responses. *J Immunol* 177:1017-1027.

111. Panjwani, N., O. Akbari, S. Garcia, M. Brazil, and B. Stockinger. 1999. The HSC73 molecular chaperone: involvement in MHC class II antigen presentation. *J Immunol* 163:1936-1942.

112. Li, P., J. L. Gregg, N. Wang, D. Zhou, P. O'Donnell, J. S. Blum, and V. L. Crotzer. 2005. Compartmentalization of class II antigen presentation: contribution of cytoplasmic and endosomal processing. *Immunol Rev* 207:206-217.

113. Acuto, O. and F. Michel. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol* 3:939-951.

114. Sharpe, A. H. and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nat Rev Immunol* 2:116-126.

115. Greenwald, R. J., G. J. Freeman, and A. H. Sharpe. 2005. The B7 family revisited. *Annu.Rev Immunol* 23:515-548.

116. Erlandsson, L., R. Blumenthal, M. L. Eloranta, H. Engel, G. Alm, S. Weiss, and T. Leanderson. 1998. Interferon-beta is required for interferon-alpha production in mouse fibroblasts. *Curr.Biol* 8:223-226.

117. Muller, U., U. Steinhoff, L. F. Reis, S. Hemmi, J. Pavlovic, R. M. Zinkernagel, and M. Aguet. 1994. Functional role of type I and type II interferons in antiviral defense. *Science* 264:1918-1921.

118. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76:17-27.

119. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 76:34-40.

120. Mombaerts, P., J. Iacomini, R. S. Johnson, K. Herrup, S. Tonegawa, and V. E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* 68:869-877.

121. Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A. M. Stall, and . 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68:855-867.

122. Hunt, C. R., D. J. Dix, G. G. Sharma, R. K. Pandita, A. Gupta, M. Funk, and T. K. Pandita. 2004. Genomic instability and enhanced radiosensitivity in Hsp70.1- and Hsp70.3-deficient mice. *Mol.Cell Biol* 24:899-911.

123. Bendelac, A., R. D. Hunziker, and O. Lantz. 1996. Increased interleukin 4 and immunoglobulin E production in transgenic mice overexpressing NK1 T cells. *J Exp.Med* 184:1285-1293.

124. Mendiratta, S. K., W. D. Martin, S. Hong, A. Boesteanu, S. Joyce, and L. Van Kaer. 1997. CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4. *Immunity* 6:469-477.

125. Karttunen, J., S. Sanderson, and N. Shastri. 1992. Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens. *Proc.Natl Acad.Sci.U.S A* 89:6020-6024.

126. Porgador, A., J. W. Yewdell, Y. Deng, J. R. Bennink, and R. N. Germain. 1997. Localization, quantitation, and in situ detection of specific peptide-MHC class I complexes using a monoclonal antibody. *Immunity* 6:715-726.

127. Moore, M. W., F. R. Carbone, and M. J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* 54:777-785.

128. Wilson, N. S., D. El Sukkari, G. T. Belz, C. M. Smith, R. J. Steptoe, W. R. Heath, K. Shortman, and J. A. Villadangos. 2003. Most lymphoid organ dendritic cell types are phenotypically and functionally immature. *Blood* 102:2187-2194.

129. Ostling, P., J. K. Bjork, P. Roos-Mattjus, V. Mezger, and L. Sistonen. 2007. Heat shock factor 2 (HSF2) contributes to inducible expression of hsp genes through interplay with HSF1. *J Biol Chem.* 282:7077-7086.

130. Shortman, K. and Y. J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2:151-161.

131. Le Bon, A., N. Etchart, C. Rossmann, M. Ashton, S. Hou, D. Gewert, P. Borrow, and D. F. Tough. 2003. Cross-priming of CD8(+) T cells stimulated by virus-induced type I interferon. *Nature Immunology* 4:1009-1015.

132. Wells, J. W., C. J. Cowled, D. Darling, B. A. Guinn, F. Farzaneh, A. Noble, and J. Galea-Lauri. 2007. Semi-allogeneic dendritic cells can induce antigen-specific T-cell activation, which is not enhanced by concurrent alloreactivity. *Cancer Immunol Immunother.* 56:1861-1873.

133. Nadler, S. G., M. A. Tepper, B. Schacter, and C. E. Mazzucco. 1992. Interaction of the immunosuppressant deoxyspergualin with a member of the Hsp70 family of heat shock proteins. *Science* 258:484-486.

134. Diegel, M. L., S. G. Nadler, and P. A. Kiener. 2002. In vivo administration of 15-deoxyspergulin inhibits antigen-presenting cell stimulation of T cells and NF-kappaB activation. *Int.Immunopharmacol.* 2:1451-1464.

135. Hoeger, P. H., M. A. Tepper, A. Faith, J. A. Higgins, J. R. Lamb, and R. S. Geha. 1994. Immunosuppressant deoxyspergualin inhibits antigen processing in monocytes. *J.Immunol* 153:3908-3916.

136. Batista, F. D. and N. E. Harwood. 2009. The who, how and where of antigen presentation to B cells. *Nat Rev Immunol* 9:15-27.

137. Kitajima, T., G. Caceres-Dittmar, F. J. Tapia, J. Jester, P. R. Bergstresser, and A. Takashima. 1996. T cell-mediated terminal maturation of dendritic cells: loss of adhesive and phagocytotic capacities. *J Immunol* 157:2340-2347.

138. Moulin, V., F. Andris, K. Thielemans, C. Maliszewski, J. Urbain, and M. Moser. 2000. B lymphocytes regulate dendritic cell (DC) function in vivo: increased interleukin 12 production by DCs from B cell-deficient mice results in T helper cell type 1 deviation. *J Exp.Med* 192:475-482.

139. Yu, P., Y. Wang, R. K. Chin, L. Martinez-Pomares, S. Gordon, M. H. Kosco-Vibois, J. Cyster, and Y. X. Fu. 2002. B cells control the migration of a subset of dendritic cells into B cell follicles via CXC chemokine ligand 13 in a lymphotoxin-dependent fashion. *J Immunol* 168:5117-5123.

140. Matthews, A. G. and M. A. Oettinger. 2009. RAG: a recombinase diversified. *Nat Immunol* 10:817-821.

141. Rizzitelli, A., R. Berthier, V. Collin, S. M. Candeias, and P. N. Marche. 2002. T lymphocytes potentiate murine dendritic cells to produce IL-12. *J Immunol* 169:4237-4245.

142. Crowley, M. T., C. R. Reilly, and D. Lo. 1999. Influence of lymphocytes on the presence and organization of dendritic cell subsets in the spleen. *J Immunol* 163:4894-4900.

143. Shreedhar, V., A. M. Moodycliffe, S. E. Ullrich, C. Bucana, M. L. Kripke, and L. Flores-Romo. 1999. Dendritic cells require T cells for functional maturation in vivo. *Immunity* 11:625-636.

144. Giodini, A., C. Rahner, and P. Cresswell. 2009. Receptor-mediated phagocytosis elicits cross-presentation in nonprofessional antigen-presenting cells. *Proc.Natl Acad.Sci.U.S A* 106:3324-3329.

145. Burgdorf, S., A. Kautz, V. Bohnert, P. A. Knolle, and C. Kurts. 2007. Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science* 316:612-616.

146. Lu, J. H., B. K. Teh, L. Wang, Y. N. Wang, Y. S. Tan, M. C. Lai, and K. B. Reid. 2008. The classical and regulatory functions of C1q in immunity and autoimmunity. *Cell Mol.Immunol* 5:9-21.

147. Regnault, A., D. Lankar, V. Lacabanne, A. Rodriguez, C. Thery, M. Rescigno, T. Saito, S. Verbeek, C. Bonnerot, P. Ricciardi-Castagnoli, and S. Amigorena. 1999. Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. *J Exp.Med* 189:371-380.

148. Nimmerjahn, F. and J. V. Ravetch. 2006. Fcgamma receptors: old friends and new family members. *Immunity* 24:19-28.

149. Sancho, D., O. P. Joffre, A. M. Keller, N. C. Rogers, D. Martinez, P. Hernanz-Falcon, I. Rosewell, and Reis e Sousa. 2009. Identification of a dendritic cell receptor that couples sensing of necrosis to immunity. *Nature* 458:899-903.

150. Roark, J. H., S. H. Park, J. Jayawardena, U. Kavita, M. Shannon, and A. Bendelac. 1998. CD1.1 expression by mouse antigen-presenting cells and marginal zone B cells. *J Immunol* 160:3121-3127.

151. Bialecki, E., C. Paget, J. Fontaine, M. Capron, F. Trottein, and C. Faveeuw. 2009. Role of marginal zone B lymphocytes in invariant NKT cell activation. *J Immunol* 182:6105-6113.

152. Amano, M., N. Baumgarth, M. D. Dick, L. Brossay, M. Kronenberg, L. A. Herzenberg, and S. Strober. 1998. CD1 expression defines subsets of follicular and marginal zone B cells in the spleen: beta 2-microglobulin-dependent and independent forms. *J Immunol* 161:1710-1717.

153. Zaini, J., S. Andarini, M. Tahara, Y. Saijo, N. Ishii, K. Kawakami, M. Taniguchi, K. Sugamura, T. Nukiwa, and T. Kikuchi. 2007. OX40 ligand expressed by DCs costimulates NKT and CD4+ Th cell antitumor immunity in mice. *J Clin.Invest* 117:3330-3338.

154. Taraban, V. Y., S. Martin, K. E. Attfield, M. J. Glennie, T. Elliott, D. Elewaut, S. Van Calenbergh, B. Linclau, and A. Al Shamkhani. 2008. Invariant NKT cells promote CD8+ cytotoxic T cell responses by inducing CD70 expression on dendritic cells. *J Immunol* 180:4615-4620.

155. Akbari, O., P. Stock, E. H. Meyer, G. J. Freeman, A. H. Sharpe, D. T. Umetsu, and R. H. DeKruyff. 2008. ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. *J Immunol* 180:5448-5456.

156. Kim, H. J., H. Y. Kim, B. K. Kim, S. Kim, and D. H. Chung. 2006. Engagement of glucocorticoid-induced TNF receptor costimulates NKT cell activation in vitro and in vivo. *J Immunol* 176:3507-3515.

157. Tesciuba, A. G., R. A. Shilling, M. D. Agarwal, H. S. Bandukwala, B. S. Clay, T. V. Moore, J. V. Weinstock, A. A. Welcher, and A. I. Sperling. 2008. ICOS costimulation expands Th2 immunity by augmenting migration of lymphocytes to draining lymph nodes. *J Immunol* 181:1019-1024.

158. Platanias, L. C. 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nature Reviews Immunology* 5:375-386.

159. Beignon, A. S., M. Skoberne, and N. Bhardwaj. 2003. Type I interferons promote cross-priming: more functions for old cytokines. *Nature Immunology* 4:939-941.

160. Cho, H. J., T. Hayashi, S. K. Datta, K. Takabayashi, J. H. Van Uden, A. Horner, M. Corr, and E. Raz. 2002. IFN-alpha beta promote priming of antigen-specific CD8(+) and CD4(+) T lymphocytes by immunostimulatory DNA-based vaccines. *Journal of Immunology* 168:4907-4913.

161. Sporri, R. and Reis e Sousa. 2003. Newly activated T cells promote maturation of bystander dendritic cells but not IL-12 production. *J Immunol* 171:6406-6413.

162. Talay, O., C. H. Shen, L. Chen, and J. Chen. 2009. B7-H1 (PD-L1) on T cells is required for T-cell-mediated conditioning of dendritic cell maturation. *Proc.Natl Acad.Sci.U.S A* 106:2741-2746.

163. Javid, B., P. A. MacAry, and P. J. Lehner. 2007. Structure and function: heat shock proteins and adaptive immunity. *J.Immunol* 179:2035-2040.

164. Bezbradica, J. S., A. K. Stanic, N. Matsuki, H. Bour-Jordan, J. A. Bluestone, J. W. Thomas, D. Unutmaz, L. Van Kaer, and S. Joyce. 2005. Distinct roles of dendritic cells and B cells in Va14Ja18 natural T cell activation in vivo. *J Immunol* 174:4696-4705.

165. Sonoda, K. H. and J. Stein-Streilein. 2002. CD1d on antigen-transporting APC and splenic marginal zone B cells promotes NKT cell-dependent tolerance. *Eur.J Immunol* 32:848-857.

166. Kitamura, H., A. Ohta, M. Sekimoto, M. Sato, K. Iwakabe, M. Nakui, T. Yahata, H. Meng, T. Koda, S. Nishimura, T. Kawano, M. Taniguchi, and T. Nishimura. 2000. alpha-galactosylceramide induces early B-cell activation through IL-4 production by NKT cells. *Cell Immunol* 199:37-42.

167. Stockinger, B., T. Zal, A. Zal, and D. Gray. 1996. B cells solicit their own help from T cells. *J Exp.Med* 183:891-899.

168. Chen, S., L. C. Ndhlovu, T. Takahashi, K. Takeda, Y. Ikarashi, T. Kikuchi, K. Murata, P. P. Pandolfi, C. Riccardi, M. Ono, K. Sugamura, and N. Ishii. 2008. Co-inhibitory roles for glucocorticoid-induced TNF receptor in CD1d-dependent natural killer T cells. *Eur.J Immunol* 38:2229-2240.

169. Shilling, R. A., J. M. Pinto, D. C. Decker, D. H. Schneider, H. S. Bandukwala, J. R. Schneider, B. Camoretti-Mercado, C. Ober, and A. I. Sperling. 2005. Cutting edge: polymorphisms in the ICOS promoter region are associated with allergic sensitization and Th2 cytokine production. *J Immunol* 175:2061-2065.

170. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397:263-266.

171. Dong, C., A. E. Juedes, U. A. Temann, S. Shresta, J. P. Allison, N. H. Ruddle, and R. A. Flavell. 2001. ICOS co-stimulatory receptor is essential for T-cell activation and function. *Nature* 409:97-101.

172. www.new-science-press.com

173. www.currentprotocols.com

APPENDICES

FREQUENTLY USED ABBREVIATIONS

APCs – antigen presenting cells

Ag – antigen

Ab – antibody

APC – allophycocyanin

αGalCer – α- galactosylceramide

BCR – B cell receptor

BM – bone marrow

β_2m – β_2 microglobulin

CFSE – carboxyfluorescein succinimidyl ester

Cy – cyan

cDCs – conventional dendritic cells

DCs – dendritic cells

DSG – 15-deoxyspergualin

e.g. – for example

EDTA – ethylenediaminetetraacetic acid

ELISA – enzyme-linked immunosorbent assay

ER – endoplasmic reticulum

FACS – fluorescence activated cell sorter

FcγR – Fcγ receptor

FCS – fetal calf serum

Fig. – figure

FITC – fluorescein isothiocyanate

FSC – forward scatter characteristics

hi – high

Hsp – heat shock protein

HZI – Helmholtz Zentrum für Infektionsforschung (Helmholtz Centre for Infection Research) in Braunschweig, Germany

i.e. – this is

IFNs – type I Interferons

Ig – immunoglobulin

IL – interleukin

IMDM – Iscove's modified Dulbecco's medium

int – intermediate

i.v. – intravenously

KO – knockout

LN – lymph node

lo – low

LPS – lipopolysaccharide

MFI – mean fluorescence intensity

Mφ – macrophage

MHC – major histocompatibility complex

MIIC – MHC class II compartment

MZ – marginal zone

MZ B cells – marginal zone B cells

NKT cells – natural killer T cells

OD – optical density

ONPG – ortho-nitrophenyl-β-galactoside

OVA – ovalbumin

OVA-bio – biotynylated ovalbumin

pDCs – plasmacytoid dendritic cells

PAMPs – pathogen associated molecular patterns

PBS – phosphate buffered saline

PCR – polymerase chain reacition

PE – phycoerythrin

PI – propidium iodide

PMA – phorbol myristate acetate

PMSF – phenylmethylsulphonyl fluoride

Poly(I:C) – polyinosinic:polycytidylic acid

PVDF – polyvinylidene difluoride

qRT-PCR – quantitative real-time PCR

PRR – pathogen recognition receptors

RIPA – radio immuno precipitation assay

RAG – recombination acitvated gene

RT – room temperature

SSC – side scatter characteristics

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAP – transporter associated with antigen processing

TCR – T cell receptor

TLR – toll-like receptor

T_H – T helper cell

tg – transgenic

UV – ultraviolet

WT – wild type

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29.05.2009 Förderpreis des Arbeitskreises für Zellbiologie

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